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(54) Title: METHOD FOR DELIVERING ESTROGEN

(57) Abstract

The present invention relates to methods for the treatment or prevention of osteoporosis and the alleviation of the symptoms of menopause by the administration of an estrogen glycoside or estrogen orthoester glycoside.

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Method For Delivering Estrogen

Background of the Invention

Field of the Invention

The invention is in the field of Medicinal Chemistry.

Related Art

Menopause is defined as the final episode of menstrual bleeding in women. The term is also used colloquially to refer to the period of time encompassing the transitional period between the reproductive years up to and beyond the final episode of menstrual bleeding. For the purposes of this invention, reference to menopause will encompass this transitional period. Menopause is the result of the cessation of follicular development, which leads to a drop in the production of estradiol and other hormones. While 60% of estrogen formed in pre-menopausal women is in the form of estradiol, most of which is produced in the ovaries of ovulating women, post-menopausal ovaries produce a minimal amount of estrogen, with extraglandular tissues providing the majority of post-menopausal estrogen synthesis.

Symptoms associated with menopause include vasomotor instability (hot flashes), a decrease in breast size, atrophy of the urogenital epithelium and skin, and osteoporosis, a disease which is characterized by a marked loss of bone mass. Hot flashes may be accompanied by nervousness, anxiety, irritability and depression. Osteoporosis and the decrease in size of the female reproductive tract and breasts have been closely correlated to low levels of estrogen, while the pathology of hot flashes, though less well understood, may also be related to the decrease in estrogen production accompanying menopause.

The significant loss of bone mass which occurs at the time of menopause ultimately gives rise to osteopenia, which in turn gives rise to

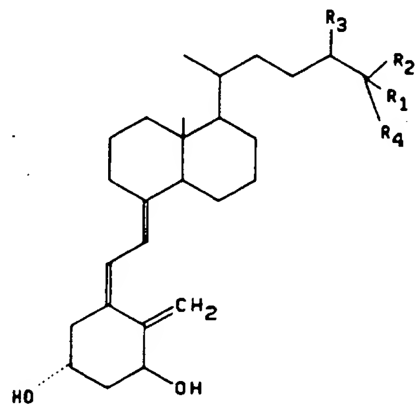
spontaneous crush fractures of the vertebrae and fractures of the long bones. This disease is generally known as postmenopausal osteoporosis and presents a major medical problem, both in the United States and most other countries where the life-span of females reaches ages of at least 60 and 70 years. Generally the disease, which is often accompanied by bone pain and decreased physical activity, is diagnosed by one or two vertebral crush fractures with X-ray evidence of diminished bone mass. It is known that this disease is accompanied by diminished ability to absorb calcium, decreased levels of sex hormones, especially estrogen and androgens, and a negative calcium balance.

Methods for treating the disease have varied considerably but to date no really satisfactory treatment is yet being practiced. For example, calcium supplementation by itself has not been successful in preventing or curing the disease. Other treatments, for which variable results have again been reported, have included a combination of vitamin D in large doses, calcium and fluoride. The primary problem with this approach is that fluoride induces structurally unsound bone, called woven bone, and in addition, produces a number of side effects such as increased incidence of fractures and gastrointestinal reaction to the large amounts of fluoride administered.

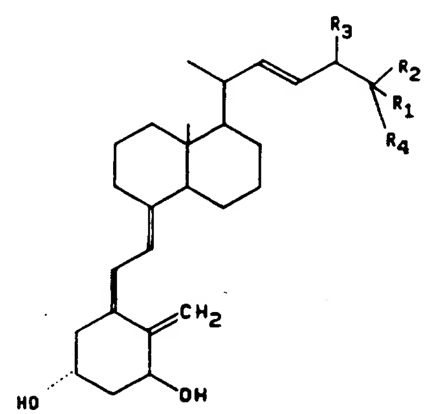
Estrogen therapy is the most commonly used treatment of menopausal symptoms. Estrogen therapy ameliorates symptoms including hot flashes and atrophy of the urogenital epithelium and skin, and has positive effects on the incidence of osteoporosis.

Premarin is the most widely prescribed estrogen that is used for the treatment of postmenopausal symptoms, including osteoporosis, in the United States. Premarin is obtained from pregnant mares' urine, and contains the sodium salts of water-soluble estrogen sulphates as well as a variety of estrogen derivatives. One of the reasons that Premarin is so popular is because of concern that unconjugated estradiol, when given orally, has a first pass effect on the liver. This effect can have potentially serious consequences including the increase of blood clotting factors which can increase the user's risk of developing blood clots.

U.S. Patent No. 4,225,596 discloses methods for treating or preventing metabolic bone disease characterized by the loss of bone mass by administering at least one compound having the formulae (I) and (II):



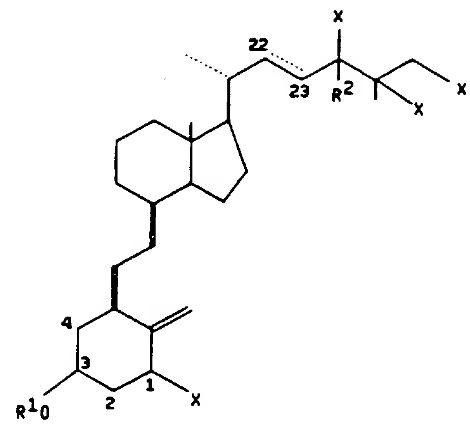
(I)



(II)

where R_1, R_2 and R_4 are each selected from the group consisting of hydrogen, hydroxyl, lower alkyl, acyl and O-alkyl and R_3 is selected from the group consisting of hydrogen, hydroxyl, keto, lower alkyl, acyl and O-alkyl.

U.S. Patent No. 4,410,515 discloses the following compounds having Formula (III) which are active in maintaining calcium and phosphorus metabolism and are useful for treating hypocalcemia in animals:



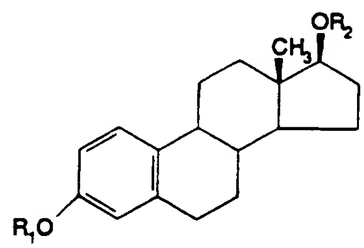
(III)

wherein the bond between positions C-22 and C-23 is single or double; R^2 is hydrogen, CH_3 or CH_2CH_3; X is selected from the group consisting of hydrogen and $-OR^1$, where R^1 is hydrogen or a straight or branched chain glycosidic residue containing 1-20 glycosidic units per residue; with the proviso that at least one of the R^1 is glycosidic residue.

Fujimoto, *et al.* (Fujimoto *et al.*, *Experientia 42*:567-568 (1988)) reported the biochemical synthesis of 17β-estradiol-3-(β-D-glucoside) and 17-(α-D-glucoside) by incubation of 17β-estradiol with ovarian tissues from a silkworm. Similarly, incubation of 17α-estradiol 3-glucuronide with rabbit liver microsomes in the presence of uridine diphosphate glucose results in the synthesis of 17α-estradiol-17-(β-glucoside). (Williamson *et al.*, *Biochemistry 8*:45299-4304 (1969)). When 17β-estradiol-17-glucoside and 17α-estradiol-17-glucoside were injected into normal women, the 3-glucuronide derivatives of each compound were recovered in the urine (Williamson *et al.*, *Canadian J. Biochemistry 50*:958-962 (1972)).

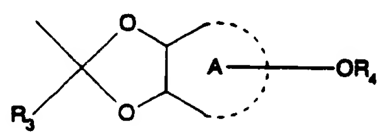
Summary of the Invention

The present invention relates to a method for treating or preventing osteoporosis, as well as a method for alleviating the symptoms of menopause, in an animal having osteoporosis or susceptible to osteoporosis, or undergoing or having undergone menopause, comprising administering to the animal an effective amount of a compound having the Formula (IV):



(IV)

wherein R_1 and R_2 are independently hydrogen or a straight or branched chain glycosidic residue containing 1-20 glycosidic units per residue, or R_1 or R_2 is an orthoester glycoside moiety of the Formula (V):



(V)

wherein A represents a glycofuranosyl or glycopyranosyl ring;

R_3 is hydrogen, lower alkyl (C_1-C_4), aralkyl (C_7-C_{10}), or aryl, with the proviso that aryl is phenyl or phenyl substituted by chloro, fluoro, bromo, iodo, lower C_1-C_4 alkyl, C_1-C_4 alkoxy; or naphthyl;

R_4 is hydrogen or a straight or branched chain glycosidic residue containing 1-20 glycosidic units per residue;

with the further proviso that at least one of R_1 and R_2 is either a glycosidic residue or an orthoester glycoside moiety.

Brief Description of the Figures

Figure 1A shows a reverse phase HPLC (2% H_2O/methanol, 1.5 ml/min) of ^{3}H-vitamin D_3-glucoside after incubation with rat intestinal homogenate for 3 hours at 37°C. No ^{3}H-vitamin D_3 was formed.

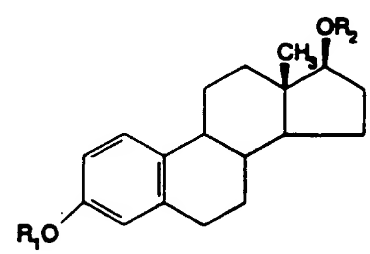
Figure 1B shows a reverse phase HPLC (2% H_2O/methanol, 1.5 ml/min) of ^{3}H-vitamin D_3-glucoside after incubation with rat kidney homogenate for 3 hours at 37°C. Note the formation of ^{3}H-vitamin D_3.

Figures 2A and 2B show the serum vitamin D concentration in human subjects after 3250 nmoles of either vitamin D_3 (2A) or vitamin D_3-glucoside (2B) was given orally.

Figure 3 shows the sequence of reactions for the synthesis of 17β-estradiol-3β-glucoside.

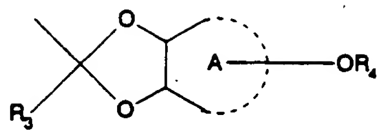
Description of the Preferred Embodiments

The invention is related to the discovery that compounds having Formula (IV) are useful in treating and preventing osteoporosis, as well as in alleviating the symptoms associated with menopause:



(IV)

wherein R_1 and R_2 are independently hydrogen or a straight or branched chain glycosidic residue containing 1-20 glycosidic units per residue, or R_1 or R_2 is an orthoester glycoside moiety of the Formula (V):



(V)

wherein A represents a glycofuranosyl or glycopyranosyl ring;

R_3 is hydrogen, lower alkyl (C_1-C_4), aralkyl (C_7-C_{10}), or aryl, with the proviso that aryl is phenyl or phenyl substituted by chloro, fluoro, bromo, iodo, lower C_1-C_4 alkyl, C_1-C_4 alkoxy; or naphthyl;

R_4 is hydrogen or a straight or branched chain glycosidic residue containing 1-20 glycosidic units per residue;

with the further proviso that at least one of R_1 and R_2 is either a glycosidic residue or an orthoester glycoside moiety.

According to the invention, the symptoms of menopause which may be alleviated include atrophy of the urogenital epithelium and skin, decreased breast size, hot flashes and osteoporosis. Any animal which experiences any of these symptoms and which may benefit from the estradiol glycosides and orthoester glycosides of Formula IV may be treated according to the present invention. Preferred animals are of course humans, in particular, pre- or post-menopausal women. When administered to a pre-menopausal woman, it is possible to prevent osteoporosis. When administered to a post-menopausal woman, it is possible to reverse the adverse consequences of osteoporosis mentioned above, and arrest the further deterioration of the bones. Administration of these compounds during menopause can alleviate the occurrence of hot flashes, decreased breast size and atrophy of the urogenital tract and skin.

By glycosidic units are meant glycopyranosyl or glycofuranosyl, as well as their amino sugar derivatives. The residues may be homopolymers, random or alternating or block copolymers thereof. The glycosidic units have free hydroxy groups, or hydroxy groups acylated with a group R_5-(C=O)-, wherein R_5 is hydrogen, lower C_{1-6} alkyl, C_{6-10} substituted or unsubstituted aryl or C_{7-16} aralkyl. Preferably, the acyl groups are acetyl or propionyl. Other

preferred groups are where R_5 is phenyl, nitrophenyl, halophenyl, lower alkyl substituted phenyl, lower alkoxy substituted phenyl, and the like or benzyl, lower alkoxy substituted benzyl and the like.

The compounds useful in the practice of the invention contain at least one glycoside or orthoester glycoside residue at positions 3 or 17. They may contain, however, two glycoside or orthoester glycoside residues simultaneously. Preferably, the glycosides or orthoester glycosides are linked through the 1-carbon to the 3- or 17-position on estradiol.

The glycosides can comprise up to 20 glycosidic units. Preferred, however, are those having less than 10, most preferred, those having 3 or less than 3 glycosidic units. Specific examples are those containing 1 or 2 glycosidic units in the glycoside residue.

The glycopyranose or glycofuranose rings or amino derivatives thereof may be fully or partially acylated or completely deacylated. The completely or partially acylated glycosides are useful as defined intermediates for the synthesis of the deacylated materials.

Among the possible glycopyranosyl structures are glucose, mannose, galactose, gulose, allose, altrose, idose, or talose. Among the furanosyl structures, the preferred ones are those derived from fructose, arabinose or xylose. Among preferred diglycosides are sucrose, cellobiose, maltose, lactose, trehalose, gentiobiose, and melibiose. Among the triglycosides, the preferred ones may be raffinose or gentianose. Among the amino derivatives are N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, N-acetyl-D-mannosamine, N-acetylneuraminic acid, D-glucosamine, lyxosylamine, D-galactosamine, and the like.

When more than one glycosidic unit is present on a single hydroxy group (i.e., di or polyglycosidic residues), the individual glycosidic rings may be bonded by 1-1, 1-2, 1-3, 1-4, 1-5 or 1-6 bonds, most preferably 1-2, 1-4 and 1-6. The linkages between individual glycosidic rings may be α or β.

The water soluble glycosidic derivatives of the aforementioned compounds may be obtained according to the general methods set disclosed

in Holick, U.S. Patent 4,410,515, the contents of which are fully incorporated by reference herein. The estradiol glycosyl orthoester compounds may be obtained according to U.S. Patent 4,521,410, the contents of which are fully incorporated by reference herein. Preferred estradiol glycosyl orthoesters are 3-estradiol-17β-(α-D-glucopyranosyl-1',2'-orthoacetate) and 17β-estradiol-3-β-glucopyranosyl-1',2'-orthoacetate.

The compounds of the invention can be administered in any appropriate pharmaceutically acceptable carrier for oral, parenteral, or topical administration. They can be administered by any means that treats or prevents osteoporosis or alleviates the symptoms of menopause in animals, especially humans. The dosage administered will be dependent upon the age, health and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment and the nature of the effect desired. For example, systemic daily dosage of 3-(β-glucopyranosyl)-17β-estradiol is from about 0.001 milligrams/kg to 100 milligrams/kg, preferably 0.01 to 1.0 milligrams per kg of body weight. Normally, from about 0.01 to 0.1 milligrams/kg per day of the glycoside or orthoester glycoside, in one or more dosages per day is effective to obtain the desired results. One of ordinary skill in the art can determine the optimal dosages and concentrations of other active estradiol, vitamin D glycoside and orthoester glycoside compounds with only routine experimentation.

The compounds can be employed in dosage forms such as tablets, capsules or powder packets, or liquid solutions, suspensions or elixirs for oral administration, as well as sterile liquid for formulations such as solutions or suspensions for parenteral use. The compounds could also be administered via topical patches, ointments, gels or other transdermal applications. In such compositions, the active ingredient will ordinarily be present in an amount of at least 1.0% by weight based upon the total weight of the composition, and not more than 90% by weight. An inert pharmaceutically acceptable carrier is preferably used. Among such carriers include 95% ethanol, vegetable oils, propylene glycols, saline buffers, etc.

It is well recognized that β-glucosidases do not exist in the small intestine because, if they did, humans would be able to digest cellulose as do ruminants. A study was conducted whereby ^3H-vitamin D_3-3β-glucoside was incubated with intestine and kidney homogenates. It was found that only the kidney homogenate was capable of removing the glucoside from vitamin D_3. None of the vitamin D_3-3β-glucoside was metabolized in the intestinal homogenates. *See* Figure 1. Therefore, the same results for estradiol-3β-glucoside would be expected since vitamin D and estradiol are both fat soluble sterols.

Since the small intestine does not contain β-glucosidases, it would not be expected that the liver would receive free estradiol coming from the intestine after the oral ingestion of estradiol-3-(β-glucoside). Thus, this would lessen the first pass effect of estradiol-3-(β-glucoside) when compared to estradiol itself.

In vivo rat studies have shown that when estradiol-3-(β-glucoside) was given orally for two weeks to ovariectomized rats, estradiol was found in the circulation (Table 1). Thus, the non-specific β-glucosidases found in peripheral tissues appear to be responsible for the de-conjugation of estradiol-3-(β-glucoside) to estradiol. As shown in Figure 1, a study with the vitamin D-3β-glucoside as a model compound was conducted which shows that β-glucosidase(s) in the kidney are able to de-conjugate the β-glucoside from the vitamin D-3β-glucoside.

One of the potential side-effects of giving estradiol orally is its first pass biologic effect on the liver especially as it relates to increasing the synthesis of proteins associated with blood clotting. Since there is evidence that estradiol-3-(β-glucoside) does not interact with the estradiol receptor, it would be expected that the estradiol-3-(β-glucoside) would not have the same potency of activity in increasing blood clotting factors in the liver as estradiol itself.

De-conjugation of estradiol-3-(β-glucoside) to estradiol will occur slowly in peripheral tissues, and therefore, will maintain circulating levels of

estradiol at a more physiologic level for a longer period of time. A study in humans with the vitamin D-3β-glucoside was conducted as a model compound. The blood level of vitamin D after an equimolar dose of vitamin D_3 and vitamin D_3-3β-glucoside was compared. As can be seen in Figure 2, the blood levels of vitamin D_3 resulting from the de-conjugation of its glucoside is about 30% of that achieved when the same dose of vitamin D_3 was given orally. Thus, from this single dose vitamin D-glucoside study, there would not appear to be any hazard to the prolonged duration of action of the estradiol resulting from the slow de-conjugation of the estradiol-3-(β-glucoside). A study in ovariectomized rats was also conducted to determine the blood level of estradiol and estrone after receiving three different doses of either estradiol or estradiol-3-(β-glucoside) orally each day for two weeks. As can been seen in Table 1, the circulating estradiol levels in the rats receiving either 1, 10 or 15 nmol of estradiol-3-(β-glucoside) were comparable or lower to the rats receiving the same doses of estradiol. Thus, estradiol-3-(β-glucoside) will increase circulating concentrations of estradiol.

Based on the results in rats and humans with the model vitamin D-3β-glucoside and experience with giving estradiol-3-(β-glucoside) to ovariectomized rats, there is no indication of potential additive or multiplicative toxicity. Indeed, in the study where oral administration of estradiol-3-(β-glucoside) to ovariectomized rats was evaluated for a period of two weeks, it was found that the β-estradiol levels were either no different or lower compared to the animals that received unconjugated estradiol (Table 1). No untoward side-effects or vaginal bleeding was noted in either group of rats.

Having now generally described this invention, the same will be understood by reference to the following examples which are provided herein for purposes of illustration only and are not intended to be limiting unless otherwise specified.

Example 1

The synthesis of 17β-estradiol-3-(β-glucoside)

17β-Estradiol-3-β-glucoside was synthesized as outlined in Figure 3. Estrone was coupled with acetobromoglucose in the presence of cadmium carbonate ($CdCO_3$) as a catalyst following the procedure of Conrow and Bernstein (Conrow and Bernstein, *J. Org. Chem. 36*:863-870 (1971)). The estrone 3β-2'3',4',6'-tetra-O-acetyl-D-glucopyranoside (A) was the predominant product (> 60%) of the reaction and was obtained by crystallization. The melting point of the product was 212-214°C (reported mp 212-216°C) (Conrow and Bernstein, *J. Org. Chem. 36*:863-870 (1971)). The IR and NMR spectra of this product matched that of reported values (Conrow and Bernstein, *J. Org. Chem. 36*:863-870 (1971)).

The estrone conjugate (A) was reduced with sodium borohydride at 0°C in methanol-THF. This reduction resulted in the stereo specific formation of the 17β-alcohol (Hobkirk et al., *J. Clin. Endocrinol. 32*:476-480 (1971)). The product (B) was obtained in almost quantitative yield after preparative TLC purification from silica plates (2:1 hexane-ethyl acetate).

Compound B was treated with methanolic ammonia at 4°C. Removal of the solvent and trituration with water produced a white crystalline solid, mp 210-220°C. The yield of the product (C), 17β-estradiol-3β-glucoside was quantitative. The HPLC analysis of the product (C_{18}-column, 20% water in methanol, 0.5 ml/minute, monitored with a UV detector set at 254 nm) produced a single peak which demonstrated its homogeneity. Interpretation of NMR and mass spectrum data is given below.

Example 2

Physico-Chemical Analysis of 17β-Estradiol-3β-Glucoside (C)

The UV absorption spectrum for compound C in methanol had a typical spectrum for estradiol, wherein λ_{max} = 280 nm.

For the mass spectrum and NMR analysis of compound C, it was converted to its penta-O-acetyl derivative by treatment with pyridine-acetic anhydride-catalytic amount of 4,4'dimethylaminopyridine. The reaction product was homogeneous on TLC and was purified by HPLC on a silica column with 5% isopropanol in n-hexane at 1 ml/minute. The isolated compound was analyzed by FABMS and NMR:

FABMS: 662.6, 643.5, 332.3, 331.3, 288.4.

NMR: (200 MHz;$CDCl_3$ solvent, TMS internal standard) δ 7.28 (overlapped with 7.26 peak of $CHCl_3$, aromatic H); δ 6.85 (m, aromatic H, 2H), δ 5.15 (m, 4H, H-1', 2', 3', 4'), δ 4.56 (d, J=9 Hz; 1H, 17-CH); δ 4.2 (m, 2H, 6'-CH_2), δ 3.72 (m, 1H, 5'-CH); δ 2.85 (m, 2H, H-6-CH_2); δ 2.3 and 2.2 (5 singlets, 15 H, 5-OAc-protons); δ 0.75 (s, 3H, 18-CH_3).

Example 3

In vivo Rat Studies

In vivo **rat studies were carried out where estradiol-3-(β-glucoside) was given orally for two weeks to ovariectomized rats. Estradiol was detected in the circulation by radioimmunoassay, using a kit purchased from ICN Biomedical (Costa Mesa CA, 92626). The results are shown in Table 1.**

Table 1

Circulating concentration of 17β-estradiol and estrone in six ovariectomized rats per group that received an oral dose of either 17β-estradiol or 17β-estradiol-3 β-glucoside (estradiol-glc) each day for two weeks.

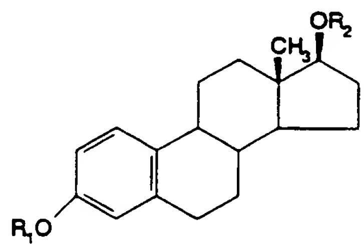
	Compounds	Dose (mmol)	Blood Concentrations	(pg/ml)
			Estradiol	Estrone
1.	control	0	3.2±0.5	22.7±3.6
2.	estradiol	1	3.5±0.7	17.8±2.0
3.	estradiol	10	8.7±2.1	18.5±2.9
4.	estradiol	15	46.8±10.4	19.8±7.0
5.	estradiol-glc	1	3.8±0.7	12.2±1.5
6.	estradiol-glc	10	7.7±2.1	12.8±1.5
7.	estradiol-glc	15	12.3±3.4	11.5±0.7

As can been seen in Table 1, the circulating estradiol levels in the rats receiving either 1, 10 or 15 nmol of estradiol-3-(β-glucoside) were comparable or lower to the rats receiving the same doses of estradiol.

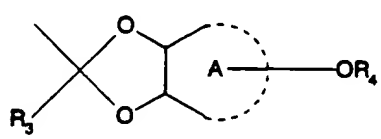
From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions without undue experimentation. All patents and publications cited herein are incorporated by reference in their entirety.

What is Claimed is:

1. A method for treating or preventing osteoporosis in an individual having osteoporosis or susceptible to osteoporosis, comprising administering to said individual an effective amount of a compound having the formula:



wherein R_1 and R_2 are independently hydrogen or a straight or branched chain glycosidic residue containing 1-20 glycosidic units per residue, or R_1 or R_2 is an orthoester glycoside moiety of the formula:



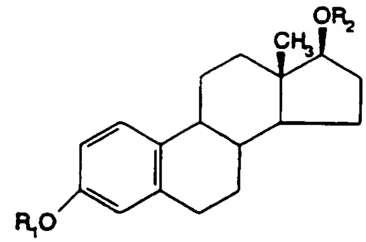
wherein A represents a glycofuranosyl or glycopyranosyl ring;

R_3 is hydrogen, lower alkyl (C_1-C_4), aralkyl (C_7-C_{10}), or aryl, with the proviso that aryl is phenyl or phenyl substituted by chloro, fluoro, bromo, iodo, lower C_1-C_4 alkyl, C_1-C_4 alkoxy; or naphthyl;

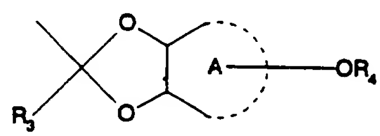
R_4 is hydrogen or a straight or branched chain glycosidic residue containing 1-20 glycosidic units per residue;

with the further proviso that at least one of R_1 and R_2 is either a glycosidic residue or an orthoester glycoside moiety.

2. A method for alleviating the symptoms of menopause in an individual undergoing or having undergone menopause, comprising administering to said individual an effective amount of a compound having the formula:



wherein R_1 and R_2 are independently hydrogen or a straight or branched chain glycosidic residue containing 1-20 glycosidic units per residue, or R_1 or R_2 is an orthoester glycoside moiety of the formula:



wherein A represents a glycofuranosyl or glycopyranosyl ring;

R_3 is hydrogen, lower alkyl (C_1-C_4), aralkyl (C_7-C_{10}), or aryl, with the proviso that aryl is phenyl or phenyl substituted by chloro, fluoro, bromo, iodo, lower C_1-C_4 alkyl, C_1-C_4 alkoxy; or naphthyl;

R_4 is hydrogen or a straight or branched chain glycosidic residue containing 1-20 glycosidic units per residue;

with the further proviso that at least one of R_1 and R_2 is either a glycosidic residue or an orthoester glycoside moiety.

3. The method of claim 1 or 2, wherein said compound is 17β-estradiol-3-(β-glucoside).

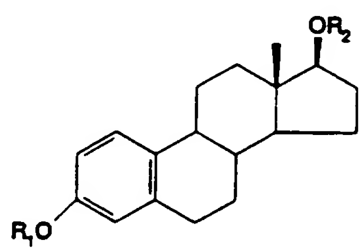
4. The method of claim 1 or 2, wherein said compound is administered in an amount ranging from about 0.01 to 10 milligrams/kg per day.

5. The method of claim 1 or 2, wherein said compound is administered as part of a pharmaceutical composition comprising a pharmaceutically acceptable carrier.

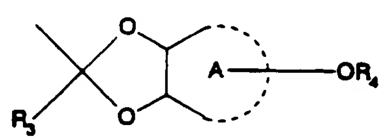
6. The method of claim 1, wherein said individual is suffering from or has suffered from menopause.

7. The method of claim 1 or 2, wherein said compound is administered to a woman prior to the onset of menopause.

8. A compound having the formula:



wherein R_1 is hydrogen, a straight or branched chain glycosidic residue containing 1-20 glucosidic units per residue, or an orthoester glycoside moiety of the formula:



wherein A represents a glycofuranosyl or glycopyranosyl ring;

R_3 is hydrogen, lower alkyl (C_1-C_4), aralkyl (C_7-C_{10}), or aryl, with the proviso that aryl is phenyl or phenyl substituted by chloro, fluoro, bromo, iodo, lower C_1-C_4 alkyl, C_1-C_4 alkoxy; or naphthyl;

R_4 is hydrogen or a straight or branched chain glycosidic residue containing 1-20 glycosidic units per residue; and

R_2 is an orthoester glycoside residue having the above formula, or a hydrogen when R_1 is an orthoesterglycoside moiety.

9. The compound of claim 8 which is 3-estradiol-17β-(α-D-glucopyranosyl-1′,2′-orthoacetate).

10. The compound of claim 8 which is 17β-estradiol-3-β-glucopyranosyl-1′,2′-orthoacetate.

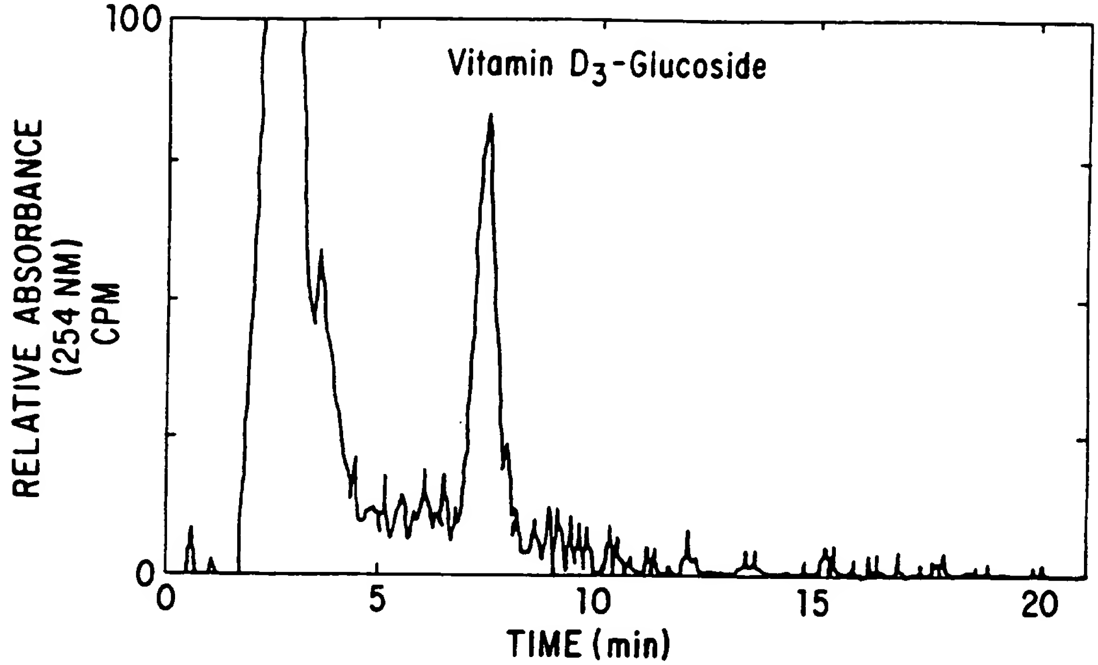

Vitamin D_3-Glucoside
RELATIVE ABSORBANCE (254 NM) CPM
TIME (min)
100
0
0
5
10
15
20

FIG. 1A

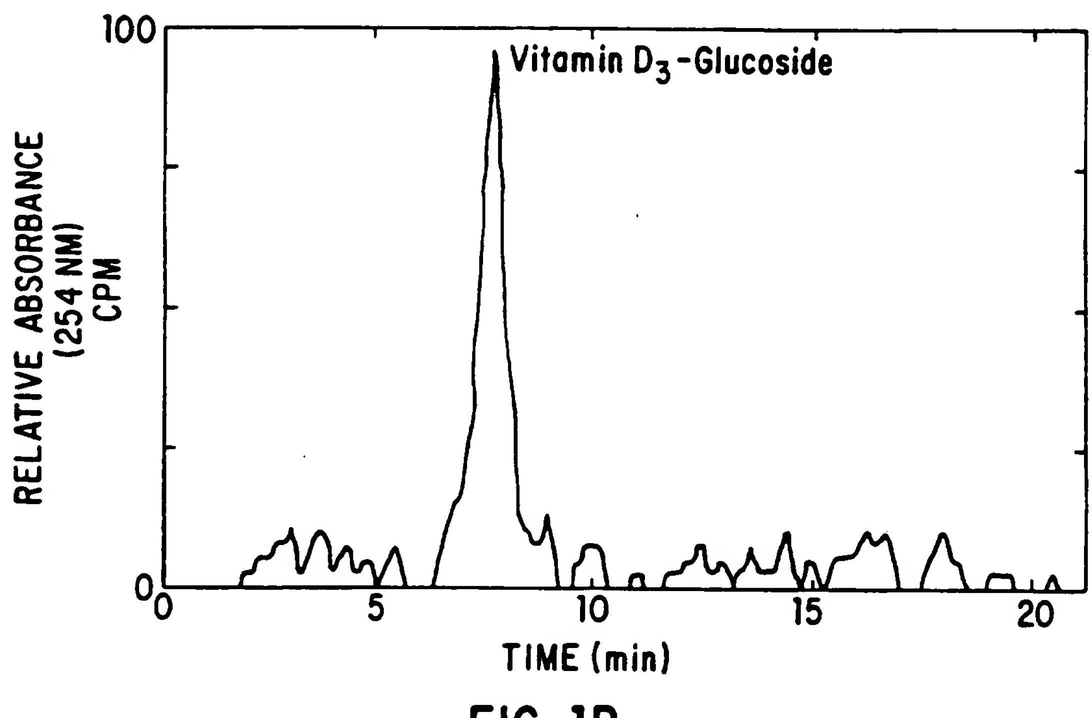

Vitamin D_3-Glucoside
RELATIVE ABSORBANCE (254 NM) CPM
TIME (min)
100
0
0
5
10
15
20

FIG. 1B

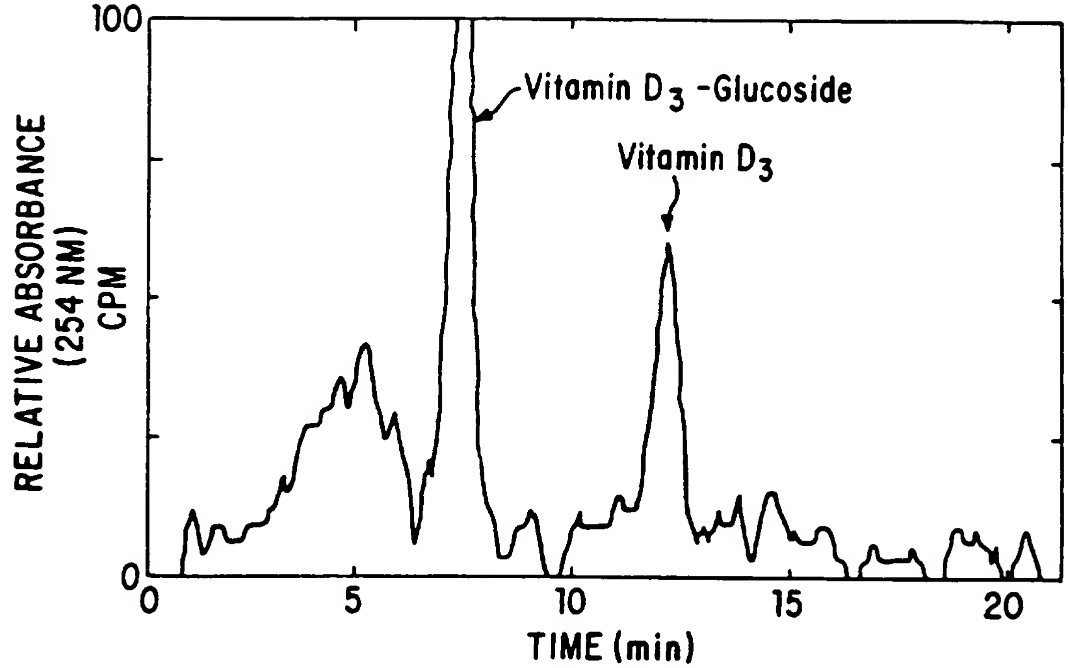

100
0
RELATIVE ABSORBANCE
(254 NM)
CPM
0
5
10
15
20
TIME (min)
Vitamin D3 -Glucoside
Vitamin D3

FIG. 1C

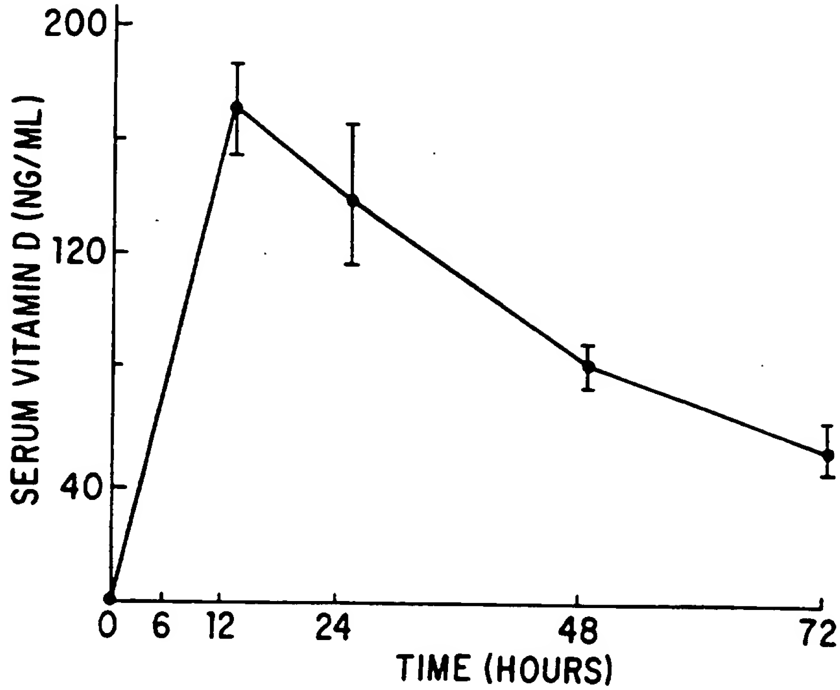


FIG. 2A

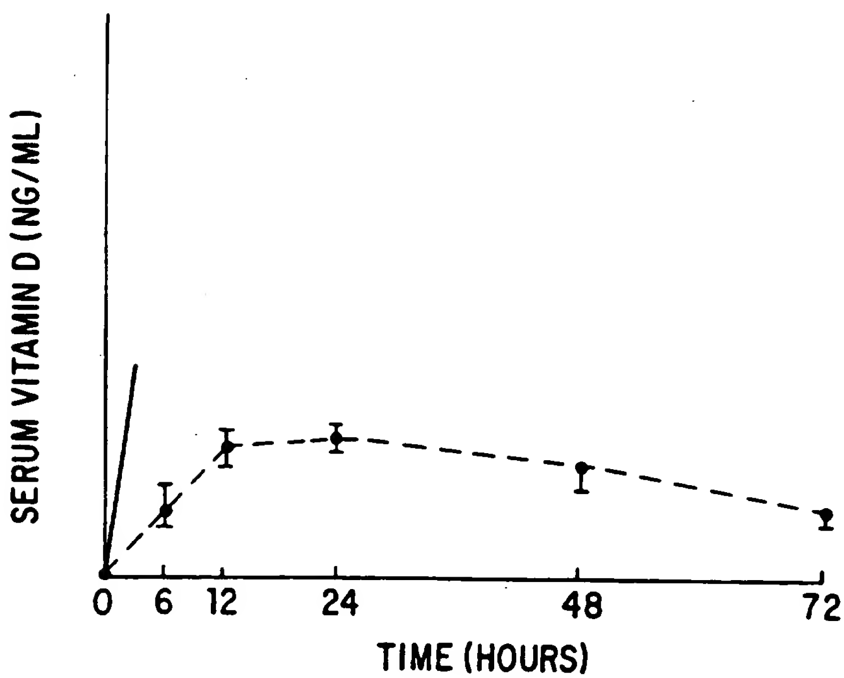


FIG. 2B

RECTIFIED SHEET (RULE 91)

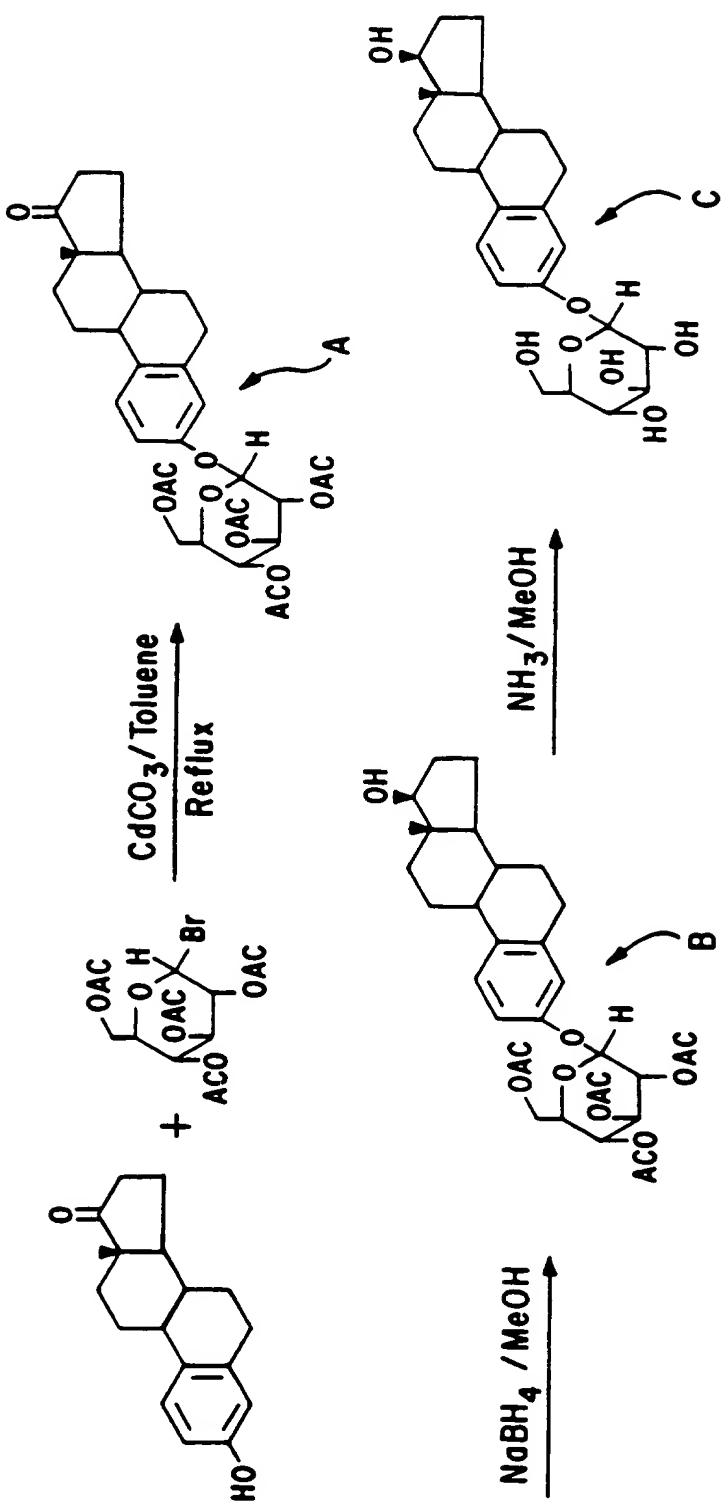


FIG. 3

RECTIFIED SHEET (RULE 91)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/09720

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/705; C07J 1/00
US CL :514/26; 536/5; 552/502
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/26; 536/5; 552/502

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

HCAPlus, BIOSIS, MEDLINE, APS
search terms: menopause, osteoporosis, estradiol [50-28-2]

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Biochemistry, Volume 8, Number 11, issued November 1969, Williamson et al., "Isolation of 17α-Estradiol 17-β-D-Glucopyranoside from Rabbit Urine, and Its Synthesis and Characterization", pages 4299-4304, see entire document.	8-10 ---- 1-7
X --- Y	Experientia, Volume 42, issued 1986, Fujimoto et al., "Characterization of 17β-estradiol 3-(β-*D*-glucopyranoside) and 17-(α-*D*-glucopyranoside) as the metabolites of 17β-estradiol in the cultured ovaries of the silkworm, *Bombyx mori*", pages 567-568, see entire document.	8-10 ---- 1-7
Y	Gilman et al., "Goodman and Gilman's The Pharmacological Basis of Therapeutics", Eighth Edition, published 1990 by Pergamon Press, Inc. (Maxwell House, Fairview Park, Elmsford, NY), pages 1390-1395, see entire document.	1-7

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
11 OCTOBER 1995

Date of mailing of the international search report
25 OCT 1995

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/09720

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US, A, 4,078,060 (BENSON ET AL.) 07 March 1978, see entire document.	1-10
A	US, A, 4,096,254 (BENSON ET AL.) 20 June 1978, see entire document.	1-10
A	US, A, 5,183,814 (DUKES) 02 February 1993, see entire document.	1-10

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(54) **25-Hydroxyvitamin D3-1a-hydroxylase and dna encoding the hydroxylase**

(57) The present invention relates to a polypeptide having 25-hydroxyvitamin D_3-1α-hydroxylase activity, being useful for the prevention, diagnosis and therapeutic treatment of adult diseases such as osteoporosis induced by the decrease of active type vitamin D_3 and catalyzing the final stage of vitamin D_3 activation; and the gene encoding the polypeptide.

In accordance with the present invention, the following can be provided; a polypeptide having 25-hydroxyvitamin D_3-1α-hydroxylase activity, DNA encoding the polypeptide, a recombinant DNA prepared by inserting the DNA in a vector, a transformant carrying the recombinant DNA, a method for preparing 25-hydroxyvitamin D_3-1α-hydroxylase by using the transformant, a method for preparing 1α, 25-dihydroxyvitamin D_3 comprising using the polypeptide having 25-hydroxyvitamin D_3-1α-hydroxylase activity, and an antibody recognizing the polypeptide.

EP 0 890 643 A2

Description

The present invention relates to a polypeptide having 25-hydroxyvitamin D_3-1α-hydroxylase activity, DNA encoding the polypeptide, a recombinant DNA prepared by inserting the DNA in a vector, a transformant carrying the recombinant DNA, a method for preparing 25-hydroxyvitamin D_3-1α-hydroxylase by using the transformant, a method for preparing 1α, 25-dihydroxyvitamin D_3 by using the polypeptide having 25-hydroxyvitamin D_3-1α-hydroxylase activity and to an antibody recognizing the polypeptide.

Active type vitamin D_3 has been known as a hormone having various biological activities such as the activity of controlling calcium metabolism, the induction of cellular differentiation, and immunomodulation.

It has been known that active type vitamin D_3 is generated from vitamin D_3 having no biological activities through the metabolism in biological organisms.

As one of the action mechanisms of active type vitamin D_3, an action mechanism through cytoplasmic receptors has been known.

It has been known that active type vitamin D_3 is essentially 1α, 25-dihydroxyvitamin D_3 wherein the positions 1α and 25 have been hydroxylated. As to the metabolic pathway for the activation, it has been known that vitamin D_3 is firstly modified into 25-hydroxyvitamin D_3 by introducing a hydroxyl group into the position 25 and the position 1α of the resulting 25-hydroxyvitamin D_3 is hydroxylated to form 1α, 25-dihydroxyvitamin D_3 [All of vitamin D_3, edited by Etsuro Ogata, Tateo Suda, and Yosuke Ogura, Kodansha Scientific, Co. (1993)].

As 25-hydroxylase gene which functions to introduce a hydroxyl group into the position 25, a gene derived from rat liver has been cloned (Japanese Published Unexamined Patent Application No.2324893/1991). Furthermore, the gene of the hydroxylase of the position 24 of vitamin D_3 has been cloned [Japanese Published Unexamined Patent Application No.207196/1992].

As an enzyme to hydroxylate the position 1α of vitamin D_3, human CYP27 has been reported [Proc. Natl. Acad. Sci., USA, 91, 10014 (1994)], but the activity of the enzyme to hydroxylate the position 1 α is a secondary activity, so the activity is very weak, which is not an essential activity. Additionally, the activity is not inducible.

It has been known that 25-hydroxyvitamin D_3-1α-hydroxylase activity is induced in the kidneys of rats and chickens fed with vitamin D_3 deficient diet [Gerontology, 42 (Supplement 1), 67-77 (1996)].

Up to now, no report has been presented yet in any of animal species, concerning the isolation of any enzyme polypeptide catalyzing the final stage of vitamin D_3 activation to hydroxylate the most significant position 1α, or the isolation of a gene encoding the polypeptide.

As a method for producing 1α, 25-dihydroxyvitamin D_3, a method comprising the use of kidney homogenates or mitochondria fractions of animals such as chicken has been known [Nature, 230, 228 (1971); J. Biol. Chem., 247, 7528 (1972); Biochemistry, 25, 5512 (1986)], but the method requires a vast amount of animal kidney or liver and demands laborious works to prepare them, so the method is insufficient and is not practical. It has been found a microorganism having activity to directly induce hydroxyl groups into the positions 1α and 25 (Japanese Published Examined Patent Application No.64678/1992), but the activity is very weak and substrate specificity is low, so it is difficult to separate the product and byproducts.

Thus, the technical problem underlying the present invention was to solve the above-mentioned problems in the prior art.

The solution to the technical problem is provided by the embodiments characterized in the claims.

Accordingly, the present invention provides a polypeptide having 25-hydroxyvitamin D_3 -1α-hydroxylase activity and a gene encoding the polypeptide. 25-hydroxyvitamin D_3-1α-hydroxylase catalyzes the final stage of vitamin D_3 activation, and is useful for prevention, diagnosis and therapeutic treatment of diseases such as osteoporosis induced by the decrease of active type vitamin D_3.

The present invention relates, in addition, to a polypeptide having 25-hydroxyvitamin D_3-1α-hydroxylase activity and DNA encoding the polypeptide, to a recombinant DNA prepared by inserting the DNA in a vector, a transformant carrying the recombinant DNA, a method for producing 25-hydroxyvitamin D_3-1α-hydroxylase or a polypeptide having 25-hydroxyvitamin D_3-1α-hydroxylase activity by using the transformant, a method for producing 1α, 25-dihydroxyvitamin D_3 by using the polypeptide having 25-hydroxyvitamin D_3-1α-hydroxylase activity and an antibody recognizing the polypeptide.

The figure shows:

Fig.1 is an HPLC chart of the identified vitamin D_3 metabolites in cells to which pcMD3R or pcDNA3 is introduced, wherein "A" shows the results of the identification of vitamin D_3 metabolites in the cells to which pcMD3R is introduced; and "B" represents the results of vitamin D_3 metabolites in the cells to which pcDNA3 is introduced, wherein (1) represents 25-hydroxyvitamin D_3; (2) represents 24, 25-dihydroxyvitamin D_3; (3) represents 10-oxo-19-nor-25-hydroxyvitamin D_3 and (4) represents α, 25-dihydroxyvitamin D_3.

As the polypeptide of the present invention, any polypeptide having 25-hydroxyvitamin D_3-1α-hydroxylase activity may be used, for example including a polypeptide having an amino acid sequence selected from amino acid sequences represented by SEQ ID NOS.1 and 2, or having an amino acid sequence in which one or more amino acid residues are deleted, substituted and/or added in the amino acid sequence of a polypeptide, the amino acid sequence being selected from amino acid sequences represented by SEQ ID NOS. 1 and 2, and having 25-hydroxyvitamin D_3-1α-hydroxylase activity.

The polypeptide having an amino acid sequence in which one or more amino acid residues are deleted, substituted or added in the amino acid sequence, the amino acid sequence being selected from amino acid sequences represented by SEQ ID NOS.1 and 2, and having 25-hydroxyvitamin D_3-1α-hydroxylase activity may be prepared according to the method described in Nucleic Acids Research, 10, 6487 (1982); Proc. Natl. Acad. Sci., USA, 79, 6409 (1982); Proc. Natl. Acad. Sci., USA, 81, 5662 (1984); Science, 224, 1431 (1984); PCT WO85/00817 (1985); Nature, 316, 601 (1985); Gene, 34, 315 (1985); Nucleic Acids Research, 13, 4431 (1985); Current Protocols in Molecular Biology, Chapter 8. Mutagenesis of Cloned DNA, John Wiley & Sons, Inc. (1989); and the like.

DNA of the present invention includes DNA encoding the polypeptide of the present invention, for example, DNA encoding the polypeptide having an amino acid sequence selected from amino acid sequences represented by SEQ ID NOS.1 and 2, DNA encoding the polypeptide having an amino acid sequence in which one or more amino acid residues are deleted, substituted or added in the amino acid sequence selected from amino acid sequences represented by SEQ ID NOS.1 and 2, and having 25-hydroxyvitamin D_3-1α-hydroxylase activity, DNA comprising a nucleotide sequence selected from SEQ ID NOS.3 and 4, or DNA hybridizable with these DNAs under stringent conditions.

In the present application, "DNA hybridizable under stringent conditions" means DNA recovered by using the DNA encoding the polypeptide having 25-hydroxyvitamin D_3-1α-hydroxylase activity as a probe through colony hybridization, plaque hybridization or Southern blot hybridization or the like, specific example of which includes DNA identified by hybridization in the presence of 0.7 to 0.1 M NaCl at 65 °C by using a filter on which a DNA prepared from colonies or plaques is immobilized and then rinsing the filter at a condition of 65 °C by using 0.1 to 2 x SSC solutions (the composition of 1 x SSC solution is as follows; 150 mM NaCl and 15 mM sodium citrate).

The hybridization can be carried out according to the method described in Molecular Cloning, A Laboratory Manual, 2-nd edition, Sambrook, Fritsch & Maniatis, eds., Cold Spring Harbor Laboratory Press (1989) (referred to as "Molecular Cloning, 2-nd edition" hereinafter), Current Protocols in Molecular Biology, Supplement 1 to 34, DNA Cloning 1: Core Techniques, A Practical Approach, Second Edition, Oxford University (1995) or the like. Hybridizable DNA includes for example DNA having homology of 60% or more, preferably 80% or more, more preferably 95 % or more to the nucleotide sequence of the DNA encoding the polypeptide having an amino acid sequence selected from amino acid sequences represented by SEQ ID NOS.1 and 2.

The antibody of the present invention includes antibodies recognizing the polypeptide described above.

In a further embodiment the present invention relates to 25-hydroxyvitamin D_3-1α-hydroxylase or a polypeptide having 25-hydroxyvitamin D_3-1α-hydroxylase activity obtainable by the method of the present invention.

Furthermore, the present invention relates to a pharmaceutical composition comprising the polypeptide, the DNA, the recombinant DNA, and/or the antibody of the present invention, and, optionally, a pharmaceutically acceptable carrier and/or diluent.

In yet another embodiment the present invention relates to a diagnostic composition comprising the polypeptide, the DNA, the recombinant DNA, and/or the antibody of the present invention.

The present invention also relates to the use of the polypeptide, the DNA, and/or the recombinant DNA of the present invention for the preparation of a pharmaceutical composition for preventing or treating a disease characterized by a decrease of active type vitamin D_3.

In a preferred embodiment of the use of the present invention, said disease is osteoporosis.

In a further embodiment the present invention relates to a kit comprising: the polypeptide, the DNA, the recombinant DNA, and/or the antibody of the present invention.

1) Preparation of cDNA library from mRNA derived from rat kidney

From tissues, for example kidney of a rat fed with vitamin D_3 deficient diet to induce 25-hydroxyvitamin D_3-1α-hydroxylase activity, mRNA [sometimes referred to as poly(A)$^+$RNA] is prepared.

Method for preparing such mRNA includes a method comprising preparing the whole RNA from the rat tissues and preparing then mRNA as poly(A)$^+$RNA by using the oligo (dT) immobilized cellulose column method [Molecular Cloning, 2-nd edition]; a method comprising directly preparing mRNA from rat tissues by using kits such as Fast Track mRNA Isolation kit manufactured by Invitrogen, Co, and Quick Prep mRNA Purification Kit, manufactured by Pharmacia, Co. and the like.

Method for preparing the whole RNA includes thiocyanate guanidine-trifluoroacetic acid cesium method [Methods in Enzymol., 154, 3 (1987)], AGPC method [Experimental Medicine, 9, 1937 (1991)] and the like.

The whole RNA and mRNA may be prepared from rat tissues with no induction of 25-hydroxyvitamin D_3-1α-hydroxylase activity by similar method described above.

By using the mRNA prepared above, a cDNA library is prepared by a conventional method.

Method for preparing the cDNA library includes for example a method for preparing a cDNA library, comprising synthesizing cDNA from the mRNA derived from the kidney resected from a rat with 25-hydroxyvitamin D_3-1α-hydroxylase activity induced, by using ZAP-cDNA synthesis kit manufactured by Stratagene, Co., cDNA Synthesis System manufactured by GIBCO BRL, Co. and the like, ligating then an adapter with a digestible site with an appropriate restriction enzyme, digesting a cloning vector λ ZAP II with the restriction enzyme, and inserting the cDNA into the digested site of the cloning vector.

As the cloning vector to prepare the cDNA library, any cloning vector capable of autonomously replicating in *Escherichia coli* K12 may be used.

The cloning vector includes for example phage vector, plasmid vector and the like, preferably including λ ZAP II described above, in addition to pUC18, pBluescript (Stratagene, Co.) and the like.

As a host microorganism, any microorganism of species *Escherichia coli* may be used, preferably including *Escherichia coli* XL1-Blue, *Escherichia coli* XL2-Blue, *Escherichia coli* DH1, *Escherichia coli* MC1000 and the like.

2) Selection of an amino acid sequence characteristic to vitamin D_3 hydroxylase

Screening a region with the amino acid sequence present in common with both the hydroxylase of the position 25 of rat vitamin D_3 [Japanese Published Unexamined Patent Application No.232493/1991] and the hydroxylase of the position 24 thereof (Japanese Published Unexamined Patent Application No.207196/1992), the amino acid sequence present in the region is selected as the amino acid sequence characteristic to the hydroxylase of vitamin D_3.

The region with the amino acid sequence includes for example adrenodoxin binding region (referred to as "Region A" hereinafter), heme binding region (referred to as "Region H" hereinafter) and the like.

3) Amplification of a partial fragment of DNA encoding 25-hydroxyvitamin D_3-1α-hydroxylase

Based on the amino acid sequence of the region selected in 2) above and with reference to the codons of rat, a sense primer and an antisense primer are designed and prepared, which are appropriate for the amplification of the DNA encoding 25-hydroxyvitamin D_3-1α-hydroxylase by polymerase chain reaction (referred to as "PCR" hereinafter).

Such primers include DNA comprising a nucleotide sequence selected from nucleotide sequences represented by SEQ ID NOS.7, 8 and 9.

Using the mRNA recovered in 1), first strand DNA is synthesized by reverse transcriptase reaction. DNA synthesis may be carried out using a cDNA synthetic kit manufactured by Stratagene, Co.

Using the first strand DNA as a template and utilizing the sense primers and antisense primers as prepared above, RT (reverse transcription)-PCR is carried out to amplify a DNA region containing a part of DNA encoding 25-hydroxyvitamin D_3-1α-hydroxylase.

Using the RT-PCR amplified fragments and 3' RACE system kit manufactured by BRL, Co., PCR amplification is carried out between the RT-PCR amplified fragment and the 3-terminal poly(A) structure to recover a longer PCR amplified fragment additionally containing the noncoding region on 3' side.

More specifically, a PCR amplified fragment containing the 3' noncoding region can be recovered by synthesizing cDNA using the mRNA recovered in 1) and the oligo dT/AUAP primer in the 3' RACE system kit manufactured by BRL, CO. and conducting PCR amplification using the DNA as a template and using the AUAP primer in the 3' RACE system kit manufactured by BRL and the RT-PCR amplified fragment.

Using 5' RACE method in the same manner, a PCR amplified fragment containing the 5' region can be recovered.

It can be confirmed that the amplified DNA fragment is a partial fragment of the DNA encoding 25-hydroxyvitamin D_3-1α-hydroxylase by the following method.

Poly(A)$^+$RNAs derived from a rat induced with 25-hydroxyvitamin D_3-1α-hydroxylase activity and a non-induced rat are individually subjected to agarose electrophoresis, and the poly(A)$^+$RNAs electrophoresed are then individually transferred onto each membrane filter in a conventional manner.

Using these membrane filters, Northern hybridization is carried out using the amplified DNA fragment as a probe.

By confirming that the amplified DNA fragment is hybridizable only when using the membrane filter prepared from the poly(A)$^+$RNA derived from the rat induced with the activity, it is revealed that the DNA fragment is a partial fragment of the DNA encoding 25-hydroxyvitamin D_3-1α-hydroxylase.

The amplified DNA fragment is then inserted into a plasmid, and the resulting plasmid can be used for nucleotide sequencing and the assay of expression specificity.

The method for inserting the fragment into a plasmid includes a method for inserting the fragment into a plasmid, comprising extracting the amplified DNA fragment from the agarose using a DNA purification kit (manufactured by Bio

Rad Co.) and ligating the fragment with a vector pCRII (manufactured by Invitrogen, Co.).

4) Selection of a clone carrying DNA encoding 25-hydroxyvitamin D_3-1α-hydroxylase

A cDNA library is screened by labeling the amplified DNA fragment and subjecting the resulting fragment to colony- or plaque hybridization in a conventional manner.

The labeling of the amplified DNA fragment can be carried out using for example DIG labeling kit (#1 175 033, manufactured by Boehringer Mannheim, Co.). More specifically, a DIG-labeled amplified DNA fragment can be recovered by PCR using the amplified DNA fragment as a template and utilizing the kit.

The plaque hybridization method includes for example the following method.

The cDNA library (phage) prepared in 1) above is spread on an agar culture medium and cultivated to a final concentration of 10,000 to 20,000 plaques per one petri dish.

Hybond N^+ membrane (manufactured by Amersham, Co.) is placed on the petri dish with plaques formed thereon to transfer the plaque DNA onto the membrane.

The transfer membrane is subject to alkali treatment (comprising for example immersing the membrane in 1.5 M NaCl, 0.5M NaOH solution) and SDS treatment (comprising for example immersing in 2 x SSC, 0.1 % SDS solution), rinsing and drying, and the resulting membrane is used for hybridization as a blotted membrane with the plaque DNA immobilized thereon.

The blotted membrane is immersed in a hybridization solution [5 x SSC, 0.1 % Sarkosyl, 0.02 % SDS, 1 % blocking reagent for hybridization (manufactured by Boehringer Mannheim, Co.)] for 5 hours, and the labeled amplified DNA fragment which has been subjected to thermal treatment is added thereto for hybridization.

After hybridization, the membrane is subject to rinsing [for example, rinsing twice in 2 x SSC and 0.1 % SDS at room temperature for 5 minutes, and rinsing twice in 0.1 x SSC and 0.1 % SDS at 60 °C for 15 minutes] and blocking (for example, blocking in 1 x blocking solution (manufactured Boehringer Mannheim Co.), 0.1 M maleic acid, 0.15 M NaCl, pH 7.5], and thereafter, the labeled amplified DNA is detected by a variable method, depending on the labeling mode of the labeled amplified DNA fragment, whereby an objective clone can be selected.

When a DNA fragment labeled with DIG is used, for example, reaction with anti-DIG antibody labeled with AP and subsequent alkali treatment [for example, immersing in 0.1 M Tris-HCl (pH 9.5), 0.1 M NaCl and 50 mM $MgCl_2$ solution] are carried out, and a plaque hybridized with the probe is screened on an X-ray film using a DIG luminescence detection kit (#1 363 514, manufactured by Boehringer Mannheim, Co.) to select a clone containing DNA encoding 25-hydroxyvitamin D_3-1α-hydroxylase.

5) Recovery of DNA encoding 25-hydroxyvitamin D_3-1α-hydroxylase

From the clone recovered by the screening procedure described above in 4), DNA is isolated in a conventional manner to recover DNA encoding 25-hydroxyvitamin D_3-1α-hydroxylase.

DNA nucleotide sequencing can be done by common nucleotide sequencing methods, for example, the dideoxy method by Sanger et. al. [Proc. Natl. Acad. Sci. USA, 74, 5463 (1977)] or by sequencing by using a nucleotide sequencer such as 373A • DNA sequencer [manufactured by Perkin Elmer, Co.].

As the gene sequence of 25-hydroxyvitamin D_3-1α-hydroxylase thus determined includes DNA comprising the sequence represented by SEQ ID NO.3 or 5.

Based on the DNA sequence thus determined by the method, an objective DNA may be prepared by chemical synthesis with a DNA sequencer. Such DNA sequencer includes a DNA sequencer based on the thiophosphite method, manufactured by Shimadzu, and a DNA sequencer Model 1392 based on the phosphoramidits method, manufactured by Perkin Elmer, Co.

The rat-derived 25-hydroxyvitamin D_3-1α-hydroxylase gene as recovered above can be used to recover 25-hydroxyvitamin D_3-1α-hydroxylase gene derived from other animals, for example, humans, by the following method.

The DNA encoding 25-hydroxyvitamin D_3-1α-hydroxylase as recovered above is labeled with α-^{32}P-dCTP by using for example Megaprime DNA labeling kit (manufactured by Amersham Co.). In the same manner as for the method described above in 1), a cDNA library is prepared from objective animal tissues, for example human kidney.

The cDNA library is screened by colony- or plaque hybridization using the labeled DNA fragment described in 4) above as a probe.

From the clone recovered through the screening, the objective DNA is isolated by the method as described in 5) above, and the nucleotide sequence is determined.

The nucleotide sequence having high homology to the nucleotide sequence of the gene of rat 25-hydroxyvitamin D_3-1α-hydroxylase is defined as DNA encoding 25-hydroxyvitamin D_3-1α-hydroxylase derived from the objective animal.

The gene includes for example human kidney-derived DNA comprising the sequence represented by SEQ ID NO.

4 or 6.

6) Production of 25-hydroxyvitamin D_3-1α-hydroxylase polypeptide

To express the DNA encoding 25-hydroxyvitamin D_3-1α-hydroxylase as recovered in 5) above in a host cell, the methods described in Molecular Cloning, 2-nd edition and Current Protocols in Molecular Biology, Supplement 1 to 34 and the like may be used.

More specifically, DNA recovered in 5) is modified into DNA fragments with appropriate lengths so that the DNA encoding 25-hydroxyvitamin D_3-1α-hydroxylase might be contained therein, by using restriction enzymes or DNases, which are then inserted into the downstream of a promoter in an expression vector, and then, the expression vector with the DNA inserted therein is introduced into a host cell appropriate for the expression vector.

Any host cell capable of expressing the objective gene may be used, including for example bacteria, yeast, animal cells and insect cells.

As the expression vector, a vector, which is autonomously replicable in the host cell or possibly inserted into the chromosome and contains a promoter at the site on which the gene of 25-hydroxyvitamin D_3-1α-hydroxylase can be transcribed, may be used.

When procaryotic cells such as bacteria are used as such host cells, it is preferable that an expression vector of 25-hydroxyvitamin D_3-1α-hydroxylase gene is autonomously replicable in the procaryotic cells and the vector is composed of a promoter, a ribosome binding sequence, DNA encoding 25-hydroxyvitamin D_3-1α-hydroxylase and a transcription termination sequence. A gene regulating the promoter may be contained in the vector.

Such expression vector includes for example pBTrp2, pBTacl, pSTac2 (all commercially available from Boehringer Mannheim, Co.), pKK2-2 (manufactured by Pharmacia, Co.) pSE280 (manufactured by Invitrogen, Co.), pGEMEX-1 (manufactured by Promega, Co.), pQE-8 (manufactured by QIAGEN, Co.), pKYP10 (Japanese Published Unexamined Patent Application No.110600/1983), pKYP200 [Agric. Biol. Chem., 48, 669 (1984)], pLSA1 [Agric. Biol. Chem., 53, 277 (1989)], pGEL1 [Proc. Natl. Acad. Sci., USA 82, 4306 (1985)], pBluescript (STRATAGENE, Co.), pTrs30 (FERM BP-5407), pTrs32 (FERM BP-5408), pGHA2 (FERM BP-400), pGKA2 (FERM BP-6798), pTerm2 (Japanese Published Unexamined Patent Application No.22979/1991, US4686191, US4939094, US5160735), pKK233-2 (manufactured by Pharmacia, Co.), pGEX (manufactured by Pharmacia, Co.), pET system (manufactured by Novagen, Co.) pSupex, pUB110, pTP5, and pC194 and the like.

Any promoter which can be expressed in host cells such as *Escherichia coli* may be used, including for example promoters derived from *Escherichia coli* and phages, for example trp promoter (Ptrp), lac promoter (Plac), P_L promoter, P_R promoter, Pletl promoter, and P_{SE} promoter; SPO1 promoter, SPO2 promoter, penP promoter and the like. Additionally, artificially designed and modified promoters, such as a promoter of two Ptrp's in series (Ptrp x 2) and tac promoter may be used.

Any ribosome binding sequence may be used, as long as the sequence may be expressed in host cells such as *Escherichia coli*. Preferably, a plasmid wherein the distance between the Shine-Dalgarno sequence and the initiation codon is adjusted to an appropriate distance (for example, 6 to 18 nucleotides) may be used.

To express 25-hydroxyvitamin D_3-1α-hydroxylase gene of the present invention, a transcription termination sequence is not necessarily required, but preferably, a transcription termination sequence is arranged immediately below the structural gene.

Exanples of the host cell include microorganisms belonging to the genus *Escherichia, Serratia, Bacillus, Brevibacterium, Corynebacterium, Microbacterium, Pseudomonas,* and the like. Specific examples include *Escherichia coli* XL1-Blue, *Eschericbia coli* XL2-Blue, *Escherichia coli* DH1, *Escherichia coli* MC1000, *Escherichia coli* KY3276, *Escherichia coli* W1485, *Escherichia coli* JM109, *Escherichia coli* HB101, *Escherichia coli* No. 49, *Escherichia coli* W3110, *Escherichia coli NY49, Seratia ficaria, Seratia fonticola, Seratia liquefaciens, Seratia marcescens, Bacillus - subtilis, Bacillus amyloliguefaciens, Brevibacterium ammoniagenes, Brevibacterium immariophilum* ATCC 14068, *Brevibacterium saccharolyticum* ATCC 14066, *Corynebacterium glutamicum* ATCC 13032, *Corynebacterium glutamicum* ATCC 14067, *Corynebacterium glutamicum* ATCC 13869, *Corynebacterium acetoacidophilum* ATCC 13870, *Microbacterium ammoniaphilum* ATCC 15354, *Pseudomonas* sp. D-0110 and the like.

As the method for introducing the recombinant vectors, any method for introducing DNA into the host cells may be used, including for example a method comprising the use of calcium ion [Proc. Natl. Acad. Sci. USA, 69, 2110 (1972)], protoplast method (Japanese Published Unexamined Patent Application No.2483942/1988), and methods described in Gene, 17, 107 (1982) and Molecular & General Genetics, 168, 111 (1979).

In case of using yeast strains as host cells, expression vectors for example YEp13 (ATCC 37115), YEp24 (ATCC 37051), YCp50 (ATCC 37419), pHS19, and pHS15 may be used.

As the promoter, any promoter which can be expressed in yeast strains may be used. For example, promoters such as PHO5 promoter, PGK promoter, GAP promoter, ADH promoter, gal 1 promoter, gal 10 promoter, heat shock protein promoter, MFα1 promoter, CUP 1 promoter and the like may be listed.

Host cells used include for example *Saccharomyces cerevisae, Shizosaccharomyces pombe, Kluyveromyces lactis, Trichosporon pullulans*, and *Schwanniomyces alluvius*.

As the method for introducing the recombinant vectors, any method for introducing DNA into yeast cells may be used, for example, electroporation method [Methods. Enzymol., 194, 182 (1990)], spheroplast method [Proc. Natl. Acad. Sci. USA, 84, 1929 (1978)], lithium acetate method [Journal of Bacteriology, 153, 163 (1983)], a method described in Proc. Natl. Acad. Sci. USA, 75, 1929 (1978) and the like.

In case of using animal cells as a host, the expression vector includes for example pcDNAI, pcDM8 (commercially available from Funakoshi, Co.), pAGE107 (Japanese Published Unexamined Patent Application No.22979/1991; Cytotechnology, 3, 133 (1990)), pAS3-3 (Japanese Published Unexamined Patent Application No.227075/1990), pCDM8 [Nature, 329, 840 (1987)], pcDNAI/Amp (manufactured by Invitrogen, Co.), pREP4 (manufactured by Invitrogen, Co.), pAGE103 [J. Biochem., 101, 1307 (1987)], and pAGE210.

As a promoter, any promoter which can be expressed in animal cells may be used, including for example a promoter of IE (immediate early) gene of cytomegalovirus (human CMV), an early promoter of SV40 or a promoter of metallothionein, a promoter retrovirus, a heat shock promoter and an SRα promoter. Additionally, the enhancer of the IE gene of human CMV may be used in combination with such promoter.

Examples of the host cell include Namalwa cell, monkey cos cell, Chinese hamster CHO cell, HST5637 (Japanese Published Unexamined Patent Application No.299/1988) and the like.

As the method for introducing recombinant vector into animal cells, any method for introducing DNA into animal cells may be used, e.g., electroporation method [Cytotechnology, 3, 133 (1990)], calcium phosphate method [Japanese Published Unexamined Patent Application No.227075/1990], lipofection method [Proc. Natl. Acad. Sci., USA, 84, 7413 (1987)] and the method described in Virology, 52, 456 (1973)]. The preparation of a transformant and cultivation of the transformant may be carried out according to the method described in Japanese Published Unexamined Patent Application No.227075/1990 or Japanese Published Unexamined Patent Application No.257891/1990.

In case of using insect cells as a host, the protein may be expressed according to the methods described in Baculovirus Expression Vectors, A Laboratory Manual, W. H. Freeman and Company, New York, 1992; Current Protocols in Molecular Biology, Supplement 1-38(1987-1997); Bio/Technology, 6, 47 (1988) and the like.

More specifically, the protein can be expressed by co-introduction of the transfer vector containing interest gene and helper DNA fragment of baculovirus into an insect cell to recover a recombinant virus in the supernatant of the culture of the insect cell and infecting an insect cell with the recombinant virus.

The transfer vector for gene introduction to be used in the method includes for example pVL1392, pVL1393, pBlueBacIII (all manufactured by Invitrogen, Co.) and the like.

As the helper DNA fragment of baculovirus, for example, Autographa californica nuclear polyhedrosis virus, which is a virus infecting insects of the family Barathra, may be used.

As such insect cell, *Spodoptera frugiperda* oocytes Sf9 and Sf21 [Baculovirus Expression Vectors, A Laboratory Manual, W. H. Freeman and Company, New York, 1992], *Trichoplusia ni* oocytes High 5 (manufactured by Invitrogen Co.) and the like, may be used.

The method for co-introducing the above-described transfer vector containing interest gene and the helper DNA fragment of baculovirus into insect cells to prepare the recombinant virus includes for example calcium phosphate method (Japanese Published Unexamined Patent Application No.227075/1990), lipofection method [Proc. Natl. Acad. Sci. USA, 84, 7413 (1987)], and the like.

As the expression method of the gene, secretory production and expression of fused protein may be carried out according to the method described in Molecular Cloning, 2-nd edition and the like, in addition to direct expression.

When the gene is expressed in yeast, animal cells or insect cells, a glycosylated protein can be obtained.

The transformant thus obtained is cultivated in a culture medium to form polypeptide of the present invention in the culture, and the formed polypeptide is recovered from the culture, whereby the polypeptide of the present invention can be produced. The transformant of the present invention is cultivated in a culture medium according to a conventional method for use in cultivating hosts.

As the culture medium to cultivate a transformant recovered by using procaryotic organisms such as *Escherichia coli* or eucaryotic organisms such as yeast, any natural culture medium or any synthetic culture medium may be used, so long as it contains carbon sources, nitrogen sources, inorganic salts and the like which can be assimilated by the organisms.

Any carbon source which can be assimilated by the organisms may be used, including carbohydrates such as glucose, fructose, sucrose, molasses containing them, starch and starch hydrolysates; organic acids such as acetic acid and propionic acid; alcohols such as ethanol and propanol.

As such nitrogen sources, ammonia; ammonium salts of inorganic acids or organic salts, such as ammonium chloride, ammonium sulfate, ammonium acetate, and ammonium phosphate; other nitrogen containing compounds; peptone; meat extract; yeast extract; corn steep liquor; case in hydrolysates; soy bean meal; soy bean meal hydrolysates; various fermentation products, and digested products thereof, may be used.

As the inorganic substances, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, magnesium phosphate, magnesium sulfate, sodium chloride, ferrous sulfate, manganese sulfate, copper sulfate, calcium carbonate and the like, may be used.

Cultivation is generally carried out under aerobic conditions, for example, by shaking culture or spinner culture under aeration. The cultivation is carried out at 15 to 40 °C for 16 hours to seven days at pH 3.0 to 9.0. The pH is adjusted with an inorganic or organic acid, an alkali solution, urea, calcium carbonate, ammonia and the like.

During cultivation, antibiotics such as ampicillin and tetracycline may be added to the culture medium, if necessary.

For cultivating microorganisms transformed with an expression vector prepared using an inducible promoter, an inducer may be added to the culture medium, if necessary. For cultivating microorganisms transformed with an expression vector prepared using lac promoter, for example, isopropyl-β-D-thiogalactopyranoside may be added to the medium; for cultivating microorganisms transformed with an expression vector prepared using trp promoter, for example, indole acrylic acid may be added to the medium.

As the culture medium for cultivating a transformant recovered by using animal cells as the hosts, RPMI 1640 culture medium [The Journal of the American Medical Association, 199, 519 (1967)], Eagle's MEM culture medium [Science, 122, 501 (1952)], Dulbecco's modified MEM culture medium [Virology, 8, 396 (1959)] , DMEM culture medium (manufactured by GIBCO BRL, Co.), 199 culture medium [Proceedings of the Society for the Biological Medicine, 73, 1 (1950)] for conventional use or culture media prepared by adding fetal calf serum and the like to these culture media, may be used.

Generally, cultivation is carried out in the presence of 5% CO_2 at pH 6 to 8 at 30 to 40 °C for 1 to 7 days.

During cultivation, if necessary, antibiotics such as kanamycin and penicillin may be added to the culture medium.

As the culture medium to cultivate transformants recovered using insect cells as the hosts, culture medium for general use, such as TNM-FH culture medium [manufactured by Pharmingen, Co.], Sf-900 II SFM culture medium [manufactured by Life Technologies, Co.], ExCell 400, ExCell 405 [both manufactured by JRH Biosciences, Co.], Grace's Insect Medium [Grace, T.C.C., Nature, 195, 788 (1962)] and the like, may be used.

Cultivation is carried out at pH 6 to 7 at 25 to 30 °C for 1 to 5 days.

During cultivation, if necessary, antibiotics such as gentamycin may be added to the culture medium.

To isolate and purify the polypeptide expressed by the method described above from the culture of the transformant, conventional isolation and purification methods of enzymes may be used.

When the polypeptide of the present invention is expressed in cells at its dissolved state, a purified sample of the polypeptide is obtained as follows. The cells are recovered through centrifugation after the cultivation, suspended in an aqueous buffer, and disrupted by means of ultrasonic oscillator, French Press, Manton Gaulin homogenizer, Dynomill and the like, to recover a cell-free extract. From the supernatant recovered by the centrifugation of the cell-free extract, a purified sample can be recovered by conventional isolation and purification methods of enzymes, singly or in combination, such as solvent extraction method, salting out methods with ammonium sulfate, etc., desalting method, precipitation methods with organic solvents, anion exchange chromatography by means of resins such as diethylaminoethyl (DEAE)-Sepharose, DIAION HPA-75 (manufactured by Mitsubishi Chemical Corporation); cation exchange chromatography by means of resins such as S-Sepharose FF (manufactured by Pharmacia, Co.); hydrophobic chromatography using resins such as butyl Sepharose and phenyl Sepharose; gel filtration methods using molecular sieves; affinity chromatography method; chromato-focusing method; electrophoresis methods such as isoelectric focusing; and the like.

When the polypeptide is expressed in cells in the form of an inclusion body, a purified sample of the polypeptide is obtained as follows. The cells are similarly recovered, disrupted, and centrifuged to recover a precipitation fraction, from which the polypeptide is recovered according to a conventional method, and the inclusion body of the polypeptide is solubilized with a polypeptide denaturant. The solubilized solution is diluted or dialyzed in a dilute solution at such an extent that the resulting solution does not contain any polypeptide denaturant or the polypeptide is not any more denatured at the concentration of the polypeptide denaturant, to renature the polypeptide into a normal steric configuration, from which a purified sample can be recovered according to the same isolation and purification method as described above.

In case that the polypeptide of the present invention or derivatives thereof such as a sugar modified product thereof are secreted extracellularly, the polypeptide or the derivatives thereof can be recovered from the culture supernatant. More specifically, the culture is treated by the method as described above, such as centrifugation, to recover a soluble fraction, and from the fraction, a purified sample is recovered using the isolation and purification method as described above.

Additionally, the polypeptide expressed by the above method may be prepared by chemical synthetic methods such as Fmoc method (fluorenylmethyloxycarbonyl method), tBoc method (t-butyloxycarbonyl method) and the like. Alternatively, the polypeptide can be prepared by utilizing peptide synthesizers commercially available from Sowa Trade (manufactured by Advanced chemTech, Co., USA), Perkin-Elmer Japan (manufactured by Perkin-Elmer, Co., USA), Pharmacia Biotech (manufactured by Pharmacia Biotech, Co., Sweden), Aroka (manufactured by Protein Technology

Instrument, Co., USA), KURABO (manufactured by Synthecell-Vega, Co., USA), Japan PerSeptive Limited (manufactured by PerSeptive, Co., USA), Shimadzu, Co. and the like.

7) Production of 1α, 25-dihydroxyvitamin D_3

The polypeptide having 25-hydroxyvitamin D_3 -1α-hydroxylase and 25-hydroxyvitamin D_3 are put in an aqueous medium to form 1α, 25-dihydroxyvitamin D_3 in the aqueous medium, and the formed 1α, 25-dihydroxyvitamin D_3 is recovered from the aqueous medium. Thus, 1α, 25-dihydroxyvitamin D_3 can be produced.

As a polypeptide having 25-hydroxyvitamin D_3-1α-hydroxylase activity, the polypeptide purified by the method described above in 6) and the microbial culture obtained by the method described above in 6) or a treated product of the culture obtained by treating the culture in various ways and the like, may be used.

Examples of the treated product of the culture broth include a concentrated product of the culture, a dried product of the culture, a culture supernatant obtained by centrifuging the culture, a concentrated product of the culture supernatant, an enzyme preparation obtained from the culture supernatant, cells (including microbial cells) obtained by centrifuging the culture, a dried product of the cells, a freeze-dried product of the cells, a surfactant- treated product of the cells, an ultrasonic-treated product of the cells, a mechanically disrupted product of the cells, a solvent-treated product of the cells, an enzyme-treated product of the cells, a protein fraction of the cells (fractions having 25-hydroxyvitamin D_3-1α-hydroxylase activity), an immobilized product of the cells and an enzyme preparation obtained by extraction from the cells.

The concentration of the polypeptide having 25-hydroxyvitamin D_3-1α-hydroxylase activity is 0.01 to 50 g/l, preferably 0.05 to 10 g/l, as wet cells.

The aqueous medium includes water, buffers such as phosphate salts, carbonate salts, acetate salts, borate salts, citrate salts, and Tris; and aqueous solutions containing organic solvents such as alcohols such as methanol and ethanol; esters such as ethyl acetate; ketones such as acetone; amides such as acetoamide. If necessary, surfactants such as Triton X-100 (manufactured by Nakarai Tesque, Co.) and Nonion HS204 (manufactured by Nippon Oils and Fats Co.), or organic solvents such as toluene and xylene may be added at about 0.1 to 20 g/l.

The concentration of 25-hydroxyvitamin D_3 is 0.01 to 50 g/l, preferably 0.01 to 10 g/l.

1α, 25-dihydroxyvitamin D_3 can be produced by adding polypeptide having 25-hydroxyvitamin D_3-1α-hydroxylase activity and 25-hydroxyvitamin D_3. The reaction is carried out at 15 to 80 °C, preferably 20 to 40 °C, at pH 3 to 11, preferably pH 4 to 9, for 5 minutes to 96 hours.

8) Preparation of an antibody recognizing 25-hydroxyvitamin D_3-1α-hydroxylase

A purified product of the whole length or a partial fragment of the protein obtained by the method described in the above in 6) or a peptide having a partial amino acid sequence of the protein of the present invention is used as the antigen. The antigen is administered to animal by subcutaneous, intravenous or intraperitoneal injection together with an appropriate adjuvant (for example, complete Freund's adjuvant, aluminum hydroxide gel, pertussis vaccine, or the like).

Examples of the animals used include rabbits, goats, 3- to 20-weak-old rats, mice, hamsters and the like.

Preferable dosage of antigen is 50 to 100 μg per animal.

When a peptide is used as the antigen, it is preferred to use the peptide as the antigen after binding it covalently to a carrier protein, such as keyhole limpet haemocyanin, bovine thyroglobulin or the like. The peptide used as the antigen can be synthesized using a peptide synthesizer.

Administration of the antigen is carried out 3 to 10 times at one- to two-week intervals after the first administration. A blood sample is recovered from the fundus of the eye 3 to 7 days after each administration, and the serum is tested, for example, by enzyme immunoassay (Enzyme-linked Immunosorbent Assay (ELISA), published by Igaku Shoin (1976); Antibodies - A Laboratory Manual, Cold Spring Harbor Laboratory (1988)) as to whether it is reactive with the antigen used for immunization. A non-human mammal whose serums shows a sufficient antibody titer against the antigen used for immunization is submitted for use as the supply source of serum or antibody producing cells.

A polyclonal antibody can be prepared by isolating and purifying it from the serum.

A monoclonal antibody can be prepared by preparing a hybridoma through fusion of the antibody producing cells with myeloma cells of a non-human mammal and culturing the hybridoma, or administering the hybridoma to an animal to induce ascites tumor in the animal, and then isolating and purifying it from the culture medium or ascitic fluid.

Examples of the antibody producing cells include spleen cells, lymph nodes and antibody producing cells in peripheral blood. Particularly, spleen cells are preferred.

Examples of the myeloma cells include cell lines derived from mouse, such as P3-X63Ag8-U1 (P3-UI) cell line [Current Topics in Microbiology and Immunology, 18, 1-7 (1978)], P3-NS1/1-Ag41 (NS-1) cell line [European J. Immunology, 6, 511-519 (1976)], SP2/O-Ag14 (SP-2) cell line (Nature, 276, 269-270 (1978)], P3-X63-Ag8653 (653) cell line [J. Immunology, 123, 1548-1550 (1979)], P3-X63-Ag8 (X63) cell line [Nature, 256, 495-497 (1975)] and the like, which are

8-azaguanine-resistant mouse (BALB/c) myeloma cell lines.

Hybridoma cells can be prepared in the following manner.

Antibody producing cells and myeloma cells are fused, suspended in HAT medium (normal medium supplemented with hypoxanthine, thymidine and aminopterin) and then cultured for 7 to 14 days. After the culturing, a portion of the culture supernatant is sampled and tested, for example, by enzyme immunoassay to select those which can react with the antigen but not with protein which does not contain the antigen. Thereafter, cloning is carried out by limiting dilution analysis, and a hybridoma which shows stable and high antibody titer by enzyme immunoassay is selected as monoclonal antibody producing hybridoma cells.

With regard to the method for the isolation and purification of the polyclonal antibody or monoclonal antibody, centrifugation, ammonium sulfate precipitation, caprylic acid precipitation, or chromatography using a DEAE-Sepharose column, an anion exchange column, a protein A or G column, a gel filtration column and the like may be employed alone or as a combination thereof.

9) Utilization of the polypeptide and the DNA encoding the polypeptide of the present invention and the antibody recognizing the polypeptide of the present invention

(1) The polypeptide of the present invention can be utilized for producing 1α, 25-dihydroxyvitamin D_3 as active type vitamin D_3.

(2) The whole length or partial fragments of the polypeptide of the present invention can be utilized as an antigen against the antibody recognizing 25-hydroxyvitamin D_3-1α-hydroxylase.

(3) By administering the whole length of the 25-hydroxyvitamin D_3-1α-hydroxylase or partial fragments thereof having the activity into biological organisms, diseases due to the decrease of the enzyme protein, such as osteoporosis, can be treated therapeutically.

(4) By using the DNA of the present invention, the mRNA of 25-hydroxyvitamin D_3-1α-hydroxylase gene can be detected by Northern hybridization method (Molecular Cloning, 2-nd edition), PCR method [PCR Protocols, Academic Press (1990)], and RT-PCR method and the like.

The diagnostic method for assaying the expression level of the mRNA of the gene of 25-hydroxyvitamin D_3-1α-hydroxylase by utilizing the detection method, is useful for suppressing the onset of adult diseases such as osteoporosis induced by the decrease of active type vitamin D_3 and is also effective for early diagnosis of genetic diseases due to congenital deficiency of the 25-hydroxyvitamin D_3-1α-hydroxylase gene.

According to Northern hybridization method, the expression level of mRNA is assayed on the basis of the label of a probe hybridized, for example, on the basis of the radioactivity in case of labeling with for example ^{32}P or the fluorescence in case of fluorescent labeling. The expression level of mRNA is assayed, on the basis of the fluorescence of a DNA specific fluorescent dye, for example ethidium bromide and Cyber Green 1 which is used for staining amplified fragments.

(5) The DNA of the present invention is inserted into virus vectors such as retrovirus and adenovirus and other vectors, and the resulting DNA can be used for therapeutic treatment according to gene therapy.

(6) By using the anti-25-hydroxyvitamin D_3-1α-hydroxylase antibody of the present invention, 25-hydroxyvitamin D_3-1α-hydroxylase can be detected and assayed in samples of blood, some organs, cells and the like. Specifically preferable methods therefor include ELISA method by using microtiter plates, fluorescent antibody methods, Western blot method and the like; additionally, immuno-histological staining by using pathological sections may also be utilized. Thus, the antibody of the present invention is useful for the diagnosis of diseases such as osteoporosis, due to the decrease of the expression of vitamin D_3-1α-hydroxylase, the diagnosis of the onset thereof and early prediction of the possibility of the onset thereof and the like. Similarly, the antibody is also useful as a laboratory reagent for research works for the protein.

(7) By using the antibody of the present invention, polypeptides having 25-hydroxyvitamin D_3-1α-hydroxylase activity are immuno-histologically stained, and thus, an immuno-histological staining agent containing the antibody can be provided.

(8) By using the DNA of the present invention and through the hybridization thereof with the genome DNA, the DNA in the promoter region of the gene can be cloned. By using DNA fragments in the promoter region, molecules involved in the regulation of the expression of the gene can be screened and analyzed.

The examples illustrate the invention.

When kits were used in individual procedures, experiments were progressed according to the protocols attached to the kits, unless otherwise stated specifically. Fundamental genetic manipulation techniques were according to Molecular Cloning, 2-nd edition.

Example 1

Preparation of kidney from rats fed with vitamin D_3 deficient diet

Immediately after weaning, four male SD rats were given vitamin D_3 deficient diet for 3 weeks (age 6 weeks).

DIET 11 [Suda, et. al., J. Nutrition, 100, 1049 (1970); commercially available as Purified diet for Rat from Teklad Co, Madison, WI, USA] was used as the vitamin D_3 deficient diet. The diet was vitamin D deficient and low calcium diet at a calcium content of 0.03 % and a phosphate content of 0.6 %.

Deionized water was used for supplementing the rats with water.

48 hours prior to sacrifice, 1α, 25-dihydroxyvitamin D_3 (manufactured by Calviochem, Co., CA, USA) was intravenously injected at 1 μg/rat into the rats.

After the designed dieting term was terminated, the rats were anesthetized with ether. From the abdominal aortas of the rats, blood was drawn out, and then, the rats were sacrificed to death by phlebotomy and immediately thereafter, the rats were autopsied to resect the kidneys.

The kidneys were rinsed in PBS [containing NaCl (8 g), KCl (0.2 g), $NaH_2PO_4 \cdot 12H_2O$ (2.9 g) and KH_2PO_4 (0.2 g) per one liter), and the resulting kidneys were frozen in liquid nitrogen.

As a control group, rats were given normal diet (Rat diet containing calcium (0.5 g), phosphate (0.6 g) and vitamin D_3 (200 IU) per 100 g) in a similar fashion, and then, the kidneys were prepared by the same method as described above. The resulting kidneys were used as kidneys from rats with no activity induction.

Example 2

Preparation of mRNA from rat kidneys

The kidneys prepared from the rats fed with the vitamin D_3 deficient diet and the kidneys derived from the rats fed with normal diet, weighed 0.78 g and 0.94 g, respectively, were rinsed in PBS and were then frozen in liquid nitrogen. The frozen kidneys can be stored at -80 °C.

The frozen kidneys were cut into pieces in liquid nitrogen with a wearing blender, until the tissues were hashed into sand size form. Then, the liquid nitrogen was evaporated.

The sand-like tissues were homogenized in ice cooling with a homogenizer (Digital Homogenizer; manufactured by Inouchi, Co.), while adding thereto 35 ml of 5.5 M GTC solution (containing 324.5 g of guanidine isothiocyanate, 3.7 g of sodium citrate, and 3.3 g of Sarkosyl in 500 ml) and 492 μl of 2-mercaptoethanol, and the homogenate in suspension was passed four times through an injection needle of gauge 18 arranged on a 50-ml injection cylinder.

The suspension was then transferred into a 15-ml centrifuge tube, for centrifugation at 6,000 rpm at 20 °C for 10 minutes, to recover the supernatant.

The supernatant was then overlaid in 16-ml portions on a CsTFA preparative solution [a mixture solution of CsTFA solution (100 ml) manufactured by Pharmacia, Co., 82.06 ml of 0.25 M EDTA solution (pH 7.0), and 23.09 ml of H_2O] in a 40-ml polyallomer tube for ultracentrifugation, and the tube was then ultra-centrifuged under conditions of 25,000 rpm and 18 °C for 25 hours.

After discarding the supernatant, the tube was cut at the position of about 1.5 cm from the bottom of the tube, and the resulting precipitate was dissolved in 0.6 ml of 4M GTC solution [a mixture solution of 5.5M GTC solution (4 ml), 1.5 ml of H_2O, and 56 μl of 2-mercaptoethanol].

The dissolved solution was centrifuged at 14,000 rpm for 15 seconds, to recover the supernatant.

After adding 15 μl of 1M sodium acetate and 0.45 ml of ethanol to the supernatant and thereby suspending the precipitate, the resulting suspension was centrifuged to recover the precipitate.

The precipitate was rinsed in 70 % ethanol, suspended in 1 ml of TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA-NaOH (pH 8.0)], and centrifuged at 14,000 rpm for 15 seconds, to recover the supernatant.

Adding a 2.5-fold volume of 70% ethanol to the supernatant, followed by centrifugation, the resulting precipitate was recovered.

The precipitate was rinsed in 70 % ethanol and was then dissolved in 500 μl of TE buffer.

Through the procedure, the whole RNA was recovered from the kidneys from the rats with activity induction and the rats with no activity induction, which was calculated as 639 μg and 918 μg, respectively, on the basis of the absorbance at 260 nm.

The whole RNA solution (150 μl) derived from the rats with activity induction was effected with thermal treatment at 65 °C for 5 minutes, which was immediately cooled in ice.

To the solution were added 0.5 ml of 5M NaCl and 0.15 g oligo dT cellulose (manufactured by Collaborative Research, Co., Type 3) equilibrated with TE/NaCl [10 mM Tris-HCl (pH 7.5), 500 mM NaCl], to adsorb the whole RNA onto the cellulose.

The cellulose was packed in a column, through which the TE/NaCl solution was passed for washing the column, followed by elution of mRNA with TE solution of 0.5 ml, to fractionate and collect the eluate in 200 μl-fractions. From the individual fractionated solutions, 2μl portions

were sampled, followed by addition of 1 μg/ml ethidium bromide (20 μl), to detect luminescent sampled solutions under ultraviolet irradiation.

Ethanol was added to the fractionated solutions corresponding to the luminescent sampled solutions, to recover precipitates.

The precipitates were rinsed in 80 % ethanol and suspended in TE buffer.

Through the procedures, mRNA of 14.3 μg was recovered from the kidneys of the rats with activity induction.

Example 3

Preparation of cDNA library

By using ZAP-cDNA synthesis kit (#200400) manufactured by Stratagene Co., a cDNA library was constructed according to the instruction manual attached to the kit.

By using 4 μg of mRNA derived from the rats with activity induction as prepared in Example 2, first strand DNA was synthesized through reverse-transcriptase reaction, and after RNase reaction, second strand DNA was synthesized with DNA polymerase I.

Under high temperature conditions, PfuDNA polymerase reaction was effected to make the termini of the cDNA to blunt end.

By ligating an *Eco*RI adapter fragment to the cDNA for phosphorylation and digesting the resulting cDNA with *Xho*I, a cDNA fragment with *Eco*RI-*Xho*I cleavage sites on both the termini was prepared.

The cDNA fragment was inserted into the *Eco*RI-*Xho*I site of λ ZAP II, and by subsequent packaging with Giga pace Gold Packaging Kit (manufactured by Stratagene, Co.) and infection by using *Escherichia coli* host XL1-Blue, MRF' strain and helper phage VCS257, a cDNA library was constructed.

Example 4

Selection of a clone harbouring mRNA molecule specifically expressed in the kidneys of the rats with induced activity

The amino acid sequences of the rat-derived hydroxylase of the position 25 of vitamin D_3 and the hydroxylase of the position 24 thereof were previously reported, and among the regions well preserved in these vitamin D_3 hydroxylases of the family P450, the partial amino acid sequences of the adrenodoxin binding region (region A) essential for the enzyme activity and of the hem binding region (region H) were selected, and on the basis of the DNA sequences were designed a sense primer and an antisense primer for PCR amplification of the gene in the regions.

More specifically, DNA comprising the nucleotide sequence represented by SEQ ID No.7 corresponding to the region A was used as the sense primer; and DNA comprising the nucleotide sequence represented by SEQ ID NO.8 corresponding to the region H was used as the antisense primer.

By using the ZAP-cDNA synthesis kit (#200400) manufactured by Stratagene Co. and 4 μg of the mRNA derived from the rats having activity induction, first strand DNA was synthesized with a primer random hexamer.

By using the first strand DNA as the template, the DNA comprising the nucleotide sequence represented by SEQ ID NO.7 as the sense primer and the DNA comprising the nucleotide sequence represented by SEQ ID NO.8 as the antisense primer and by utilizing RT-PCR kit manufactured by Stratagene, Co., PCR was effected.

By using DNA Thermal Cycler 480 manufactured by Perkin Elmer, Co., PCR was effected at 35 cycles, each cycle composed of 94 °C for 30 seconds, 42 °C for one minute and 72 °C for one minute.

The reaction product was analyzed by agarose gel electrophoresis, and a 255-bp amplification fragment (AH fragment) was observed. By using a DNA purification kit (manufactured by Bio Rad, Co.), the fragment was extracted from agarose, which was then inserted into pCRII vector (manufactured by Invitrogen, Co.).

From the whole RNAs derived from the rats induced with 25-hydroxyvitamin D_3-1α-hydroxylase activity and the non-induced rats were prepared poly(A)$^+$RNAs, which were then subject individually to agarose electrophoresis, to transfer the electrophoresed mRNAs onto membrane filters in a conventional manner.

By using these membrane filters, Northern hybridization was effected by using the amplified AH fragment as the probe.

The amplified AH fragment was hybridized only when the membrane filter prepared from the mRNA derived from the rats with activity induction was used.

The AH fragment had nucleotide sequences corresponding to the regions A and H.

By using the AH fragment and 3' RACE system kit manufactured by BRL, Co., a PCR amplified fragment containing

the 3' noncoding region of the DNA encoding the 25-hydroxyvitamin D_3-1α-hydroxylase was recovered by the following method.

By using the Oligo dT/AUAP primer attached to the 3' RACE system kit manufactured by BRL, Co. and 4 μg of the mRNA from the rats with activity induction as recovered in Example 2, cDNA was synthesized.

The cDNA was used as a template.

Based on the sequence of the AH fragment amplified above, DNA comprising the nucleotide sequence represented by SEQ ID NO.9 was synthesized and used as a sense primer.

The AUAP primer attached to the 3' RACE system kit manufactured by BRL, Co. was used as an antisense primer.

By using the template, the sense primer and the antisense primer, PCR was effected at 35 cycles, each cycle composed of 94 °C for one minute, 55 °C for one minute and 72 °C for 2 minutes.

The reaction product was analyzed by agarose gel electrophoresis, and an amplified fragment of 1.3 kb (A3 fragment) was observed. By using a DNA purification kit (manufactured by Bio Rad, Co.), the fragment was extracted from agarose, which was then inserted into pCRII vector.

In the same manner as for the AH fragment, the A3 fragment was specifically hybridized with the mRNA from the rats with activity induction.

The A3 fragment contained almost whole length of the AH fragment.

Example 5

Recovery of DNA encoding 25-hydroxyvitamin D_3-1α-hydroxylase

The cDNA phage library prepared in Example 3 was spread on an agar medium and cultivated to a final concentration of 10,000 to 20,000 plaques per one petri dish.

HybondN$^+$ membrane (manufactured by Amesham, Co.) was placed on each of the petri dishes with the plaques formed thereon, to transfer the plaque DNA onto the membrane. Two transcription membranes were prepared per one petri dish.

The transcription membranes were subject to alkali treatment (immersion in 1.5 M NaCl and 0.5 M NaOH) and SDS treatment (immersion in 2 x SSC and 0.1 % SDS solution), rinsed and dried, and then, the resulting membranes with plaque DNA immobilized thereon were used as blotting membranes for the following hybridization.

By using DIG labeling kit (#1 175 033; manufactured by Boehringer Mannheim, Co.) and 2 ng each of the AH fragment and A3 fragment as templates, PCR was effected, to recover DIG labeled AH fragment or A3 fragment.

PCR was effected under conditions of 30 cycles, each cycle of a process of 94 °C for one minute, 50 °C for one minute and 72 °C for one minute.

The resulting DIG labeled AH fragment and DIG labeled A3 fragment were used as the following probes.

The blotting membranes prepared above were immersed in a hybridization solution [5 x SSC, 0.1 % Sarkosyl, 0.02 % SDS, 1 % hybridization blocking solution (manufactured by Boehringer Mannheim, Co.)] at 60 °C for 5 hours, followed by addition of thermally treated DIG labeled probe (10 μl/10 ml-hybridization solution), for overnight hybridization at 65 °C.

After hybridization, the membranes were subject to rinsing (rinsing twice in 2 x SSC and 0.1 % SDS at room temperature for 5 minutes, rinsing twice in 0.1 x SSC and 0.1 % SDS at 60 °C for 15 minutes), blocking [effected by using 1 x blocking solution (manufactured by Boehringer Mannheim, Co.), 0.1M maleic acid, 0.15M NaCl, pH 7.5], reaction with AP labeled anti-DIG antibody (effected according to the protocol by Boehringer Mannheim, Co.), and alkali treatment [0.1M Tris-HCl (pH 9.5), 0.1M NaCl and 50 mM $MgCl_2$], and by using thereafter DIG luminescence detection kit (#1 363 514; manufactured by Boehringer Mannheim, Co.), plaques hybridizable with the probes were screened on an X-ray film.

By using firstly the DIG labeled AH fragment as the DIG labeled probe to select plaques hybridizable with the fragment and by subsequently using the DIG labeled A3 fragment, plaques hybridizable with the fragment were selected from the plaques described above.

The plaques selected at each stage were again inoculated on petri dishes, and then, it was confirmed that these were hybridizable. By PCR using both the primers of the region A and AUAP, additionally, it was confirmed that the plaques had the nucleotide sequence of the A3 fragment.

After screening of 35 petri dishes in total, finally, four plaques (Nos.221, 522, 411, 111) were selected.

From individual plaque clones was extracted DNA, which was then ligated to pBluescript vector by using rapid excision kit (#211204; manufactured by Stratagene, Co.), and subsequently, the nucleotide sequence of DNA inserted into the clone was analyzed by using M13 primer.

By the analysis with the clone No.221, DNA comprising a nucleotide sequence of 2469 bp was observed, as represented by SEQ ID No.5.

An open reading frame (referred to as ORF hereinafter) encoding 501 amino acids was observed in the DNA, in

which amino acid sequences believed as the hem binding region and adrenodoxin binding region in common with the P450 family protein were present.

Example 6

Expression of isolated 25-hydroxyvitamin D_3-1α-hydroxylase gene in animal cells

From the clone No.221 described in Example 5 was prepared a plasmid, which was subsequently digested with *Hind*III and *Xba*I. Expression vector pcDNA3 (manufactured by Invitrogen, Co.) for animal cells was similarly digested with *Hind*III and *Xba*I.

The cleavage fragments recovered above were individually subject to agarose electrophoresis, which were thereby separated and extracted.

The resulting DNA fragments from the vector and the inserted gene fragment were ligated together, by using a DNA ligation kit(manufactured by TaKaRa Brewery), to recover a ligated plasmid.

By using the plasmid, *Escherichia coli* strain DH5α was transformed, and thereafter, an ampicillin resistant strain was selected, from which the plasmid was extracted according to a known method.

Based on the analysis of the plasmid by restriction cleavage, it was confirmed that the plasmid inserted the objective gene. The plasmid was named pCMD3R.

By electroporation [Potter et. al., Proc. Natl. Acad. Sci. USA, 81, 716 (1984)], pCMD3R was introduced into an animal cell, to be expressed therein as follows. COS7 cell was cultivated in a DMEM culture medium (manufactured by GIBCO BRL, Co.) supplemented with 10 % FCS (fetal calf serum) in a petri dish for 2 days.

After cultivation, the cells were peeled off from the petri dish by trypsin treatment, and the cells were rinsed in PBS and then suspended in 0.5 ml of KPBS (137 mM KCl, 2.7 mM NaCl, 8.1 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, 4 mM $MgCl_2$), to a final concentration of 2 to 6.0 x 10^6/ml.

The suspension and 15 μg of pCMD3R plasmid were mixed together in a pulser cuvette (manufactured by BIO-RAD, Co.) with a groove width of 0.4 cm, and the resulting mixture was then applied to an electroporation system Gene pulser (manufactured by BIO-RAD, Co.) for pulse loading under conditions of 960 μF and 0.22 kV, to introduce the DNA into the cell.

The DNA introduced cell was suspended in 10 ml of DMEM culture medium containing 10 % FCS, for cultivation in a 5 % CO_2 incubator at 37 °C for 48 to 72 hours.

By discarding the culture in the petri dish and rinsing the cell twice in PBS, the cell was scraped off with a scraper, followed by centrifugation to collect the cell.

Example 7

Recovery of human-derived 25-hydroxyvitamin D_3-1α-hydroxylase gene

From 1.2 g of tissue resected from human kidney cancer, the whole RNA (750 μg) was recovered according to the method described in Example 2, and 9.5 μg of mRNA was recovered from the whole RNA.

By using 5 μg of the mRNA, a human cDNA library was constructed by the method described in Example 3.

According to the method described in Example 5, DNA encoding the human derived 25-hydroxyvitamin D_3-1α-hydroxylase was recovered.

The whole length of the rat vitamin D_3 hydroxylase gene of 2469 bp as isolated in Example 5 was DIG labeled according to the method described in Example 5, which was then used as a probe.

Hybridization was effected overnight in a hybridization solution containing formamide at 40 % under a condition of 42 °C.

Through the hybridization, four clones were selected.

According to the method described in Example 5, DNA was extracted from these clones, to analyze the nucleotide sequence of the DNA inserted into the clones.

The DNA had the nucleotide sequence represented by SEQ ID NO.6. In the DNA fragment was observed ORF encoding a peptide of 508 amino acids.

The peptide had an amino acid sequence in common with the rat-derived 25-hydroxyvitamin D_3-1α-hydroxylase in terms of 413 amino acid residues, and contained amino acid sequences possibly corresponding to the hem binding region and adrenodoxin binding region, commonly observed in the P450 family protein.

Additionally, the DNA sequence included a sequence of 1724 residues, which is the same as the sequence derived from rats, and therefore, it was indicated that the DNA had high homology.

Example 8

Expression of rat-derived vitamin D_3-1α-hydroxylase gene and assay of the activity

According to the method of Example 6, gene expression plasmid carrying the rat-derived vitamin D_3-1α-hydroxylase gene, namely pCMD3R, was introduced into COS-7 cell by electroporation.

The gene-introduced cells of 5 x 10^5 in number were cultivated in 10 ml of a DMEM culture medium containing 10 % FCS for 24 hours, and then, the culture medium was exchanged to a DMEM culture medium (8 ml) containing 1 % FCS, followed by addition of [26, 27-^{3}H]-25-hydroxyvitamin D_3 (manufactured by Amersham, Co.) at 2000 Bq/3 μl-methanol solution, and then, the resulting mixture was cultivated for 24 hours.

After cultivation, vitamin D_3 metabolites were extracted from the culture supernatant and the cells by the Bligh & Dyer's method [Can. J. Biochem., 37, 911 (1959)]. More specifically, the culture was transferred into a 50-ml centrifuge tube equipped with a screw cap, while 10 ml methanol was added into the petri dish, to scrape the cells with a scraper, and the cells were then transferred into the centrifuge tube. Methanol (10 ml) was again added into the petri dish, to suspend the cells remaining in the petri dish, and the resulting suspension was thoroughly transferred into the centrifuge tube.

Chloroform (10 ml) was added into the centrifuge tube for thorough mixing, followed by further addition of 10 ml of chloroform and subsequent complete re-mixing, and the resulting tube was left to stand to separate a chloroform layer from an aqueous layer.

The chloroform extract solution in the separated chloroform layer was placed in another centrifuge tube, followed by further addition of 10 ml of chloroform to the remaining aqueous layer, for mixing and extraction in the same manner, and the resulting chloroform extract solution was combined together with the previously recovered chloroform extract solution.

Distilled water was added into the chloroform extract solution to a final total volume of 60 ml, followed by addition of two drops of saturated sodium chloride solution and subsequent sufficient mixing.

The mixture solution was centrifuged, to separate the chloroform layer from the aqueous layer.

The resulting chloroform layer fraction was concentrated in nitrogen gas stream to recover the residue.

The residue was dissolved in 400 μl of a mixture solution iso-propanol/methanol/n-hexane = 6 : 6 : 88.

With HPLC system 880 PU manufactured by JASCO, Co. with TSK silica gel 150 column (4.6 x 250 mm; manufactured by Toso, Co.) arranged thereon, the resulting solution was subject to analysis under conditions such that the mixture solution iso-propanol/methanol/n-hexane = 6 : 6 : 88 was used as the mobile phase at a flow rate of 1 ml/minute. On comparison with the elution time of a standard substance, vitamin D_3 metabolites were identified.

Similarly, vitamin D_3 metabolites were identified by using a vector pcDNA3 which does not carry the gene of the present invention.

The results are shown in Fig.1.

"A" represents the analytical results of metabolites in the cells introduced with pcMD3R; and "B" represents the analytical results of metabolites in the cells introduced with pcDNA3. Because 1α, 25-hydroxyvitamin D_3 was detected only in the cells introduced with pcMD3R carrying the gene of the present invention, it was indicated that only the cells had 25-hydroxyvitamin D_3-1α-hydroxylase activity, which further indicates that the gene of the present invention encodes 25-hydroxyvitamin D_3-1α-hydroxylase.

In accordance with the present invention, the following can be provided; a polypeptide having 25-hydroxyvitamin D_3-1α-hydroxylase activity, being useful for the prevention, diagnosis and therapeutic treatment of adult diseases such as osteoporosis induced by the decrease of active type vitamin D_3, DNA encoding the polypeptide, a recombinant DNA prepared by inserting the DNA in a vector, a transformant carrying the recombinant DNA, a method for preparing 25-hydroxyvitamin D_3-1α-hydroxylase by using the transformant, a method for preparing 1α, 25-dihydroxyvitamin D_3 by using the polypeptide having 25-hydroxyvitamin D_3-1α-hydroxylase activity, and an antibody recognizing the polypeptide.

SEQUENCE LISTING

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6-1, Ohtemachi 1-chome,Chiyoda-ku, Tokyo 100, Japan
<120> 25-HYDROXYVITAMIN D_3-1α-HYDROXYLASE AND DNA ENCODING THE HYDROXYLASE

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Claims

1. A polypeptide comprising an amino acid sequence selected from the amino acid sequences represented by SEQ ID NOS.1 and 2, or

   a polypeptide having 25-hydroxyvitamin D_3-1α-hydroxylase activity and comprising an amino acid sequence which deviates from the amino acid sequences represented by SEQ ID NOS: 1 or 2 by the deletion, substitution and/or addition of one or more amino acid residues.

2. A DNA encoding a polypeptide according to claim 1 or

   a DNA which hybridizes with said DNA under stringent conditions.

3. The DNA according to claim 2, wherein the DNA is DNA comprising a nucleotide sequence selected from nucleotide sequences represented by SEQ ID NOS.3 and 4.

4. A recombinant DNA prepared by inserting the DNA according to claim 2 or 3 into a vector.

5. A transformant carrying a recombinant DNA according to claim 4.

6. A method for producing 25-hydroxyvitamin D_3-1α-hydroxylase or a polypeptide having 25-hydroxyvitamin D_3-1α-hydroxylase activity, comprising:

   cultivating the transformant according to claim 5 in a medium to produce 25-hydroxyvitamin D_3-1α-hydroxylase in the culture; and
   recovering said 25-hydroxyvitamin D_3-1α-hydroxylase from the resulting culture.

7. A method for producing 1α, 25-dihydroxyvitamin D_3, comprising:

   putting the polypeptide according to claim 1 and 25-hydroxyvitamin D_3 in an aqueous medium to produce 1α, 25-dihydroxyvitamin D_3 in the aqueous medium; and
   recovering said 1α, 25-dihydroxyvitamin D_3 from the aqueous medium.

8. An antibody recognizing the polypeptide according to claim 1.

9. A method for immunologically detecting a polypeptide having 25-hydroxyvitamin D_3-1α-hydroxylase activity, using the antibody according to claim 8.

10. An immuno-histological staining method comprising using an antibody according to claim 8.

11. An immuno-histological staining agent containing an antibody according to claim 8.

12. 25-hydroxyvitamin D_3-1α-hydroxylase polypeptide or a polypeptide having 25-hydroxyvitamin D_3-1α-hydroxylase activity obtainable by the method of claim 6.

13. A pharmaceutical composition comprising the polypeptide of claim 1 or 12, the DNA of claim 2 or 3, and/or the recombinant DNA of claim 4, or the antibody of claim 8, and, optionally, a pharmaceutically acceptable carrier and/or diluent.

14. A diagnostic composition comprising the polypeptide of claim 1 or 12, the DNA of claim 2 or 3, the recombinant DNA of claim 4, and/or the antibody of claim 8 and optionally suitable means for detection.

15. Use of the polypeptide of claim 1 or 12, the DNA of claim 2 or 3, and/or the recombinant DNA of claim 4 for the preparation of a pharmaceutical composition for preventing or treating a disease characterized by a decrease of active type vitamin D_3.

16. The use of claim 15, wherein said disease is osteoporosis.

17. A kit comprising:

(a) the polypeptide of claim 1 or 12;
(b) the DNA of claim 2 or 3;
(c) the recombinant DNA of claim 4; and/or
(d) the antibody of claim 8.

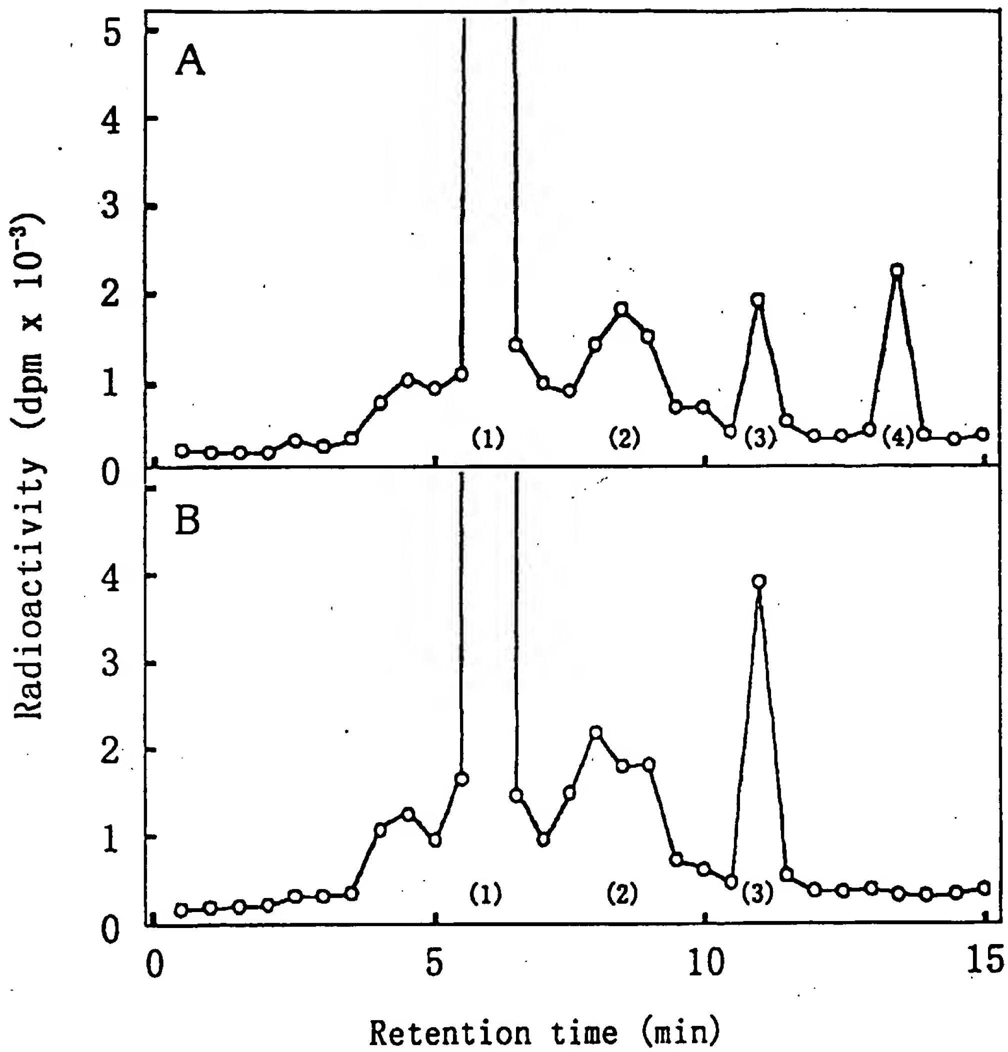
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Fig. 1